THE COLON GROUP OF BACTERIA.*†‡

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INTRODUCTION.

The presence of gas-forming bacteria in milk is usually considered an indication of fecal contamination. This is based on the assumption that all, or nearly all, of the gas-forming bacteria likely to be detected in milk by the ordinary methods of study are of the group having their habitat in the digestive tract of warm-blooded animals and finding little opportunity for multiplication under other conditions. In many board of health laboratories the determination of gas formers of the colon type is one of the routine examinations of milk, and the milk is judged largely by the results of this test.

When milk is used for cheese making the gas-forming bacteria become of economic importance because the gassy fermentation may be carried to the cheese and the product damaged by the bad flavors accompanying gassy fermentations as well as by the appearance of the cheese.

Notwithstanding the importance of the group, our knowledge of the gas-producing bacteria is very fragmentary and confusing. This knowledge has served a very useful purpose, but the time has come when, to make real progress, it is essential that we have some exact information on the various groups of bacteria concerned in the fermentations of milk, their origin, the exact nature of the changes they produce, and their relation to one another. We need

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‡ Some months after the preparation of the manuscript of this paper, appeared Frieber's article, "Die Bedeutung der Gasabsorption in der Bakteriologie," *Centralbl. f. Bakteriol.*, Abts I, *Orig.*, 1913, 69, p. 437 We are pleased to note the substantial agreement between our judgment and that of Frieber in regard to the untrustworthy nature of almost all the bacteriological gas analyses recorded in the literature. Some of the literature which we have mentioned has been covered in greater detail by Frieber, but our manner of dealing with it will be found to be a valuable supplement to Frieber's treatment.

In regard to Frieber's improvement upon the Hofstädter apparatus we must conclude, both from a theoretical consideration and from a study of the experimental results, that it still possesses defects which militate against its value for exact work.

more exact knowledge of the physiological process by which bacteria change the appearance and composition of the medium in which they live. This can come only through laborious and extensive investigations covering a large number of typical cultures. To this end many of the slipshod methods which have found their way into bacteriological technic must be displaced by the exact methods of the chemist. This is especially evident in the biochemical tests, the reduction of nitrates, the digestion of proteins, the formation of indol, and the fermentation of carbohydrates and other test substances which have been generally used in the differentiation of bacterial species.

In but few of the tests has exact analysis an honorable place. This is unfortunate. So long as an inaccurate method is employed the observer is inevitably induced to become careless, and he is tempted to rely upon the result of a statistical treatment of many determinations, forgetting the proper relationship between mathematics and data expressed in John Hopkinson's famous sentence: "We must remember that we cannot get more out of the mathematical mill than we put into it, altho we may get it in a form infinitely more useful for our purpose." But perhaps the most unfortunate consequence of the collection of inaccurate data is that the wide discrepancies destroy that faith in the definiteness of physiological processes which is the inspiration of those who hope to see bacteriology become a more exact science.

Qualitative differences, such as the fermentation of lactose by one organism and the failure of another, are doubtless distinct and positive in most cases. And yet the end products formed in the fermentation of carbohydrates by certain large classes of bacteria display a significant monotony, which should make one pause to consider whether some of the reactions involved are not common to all, whether some qualitative distinctions are not false, and whether any system of classification which pretends to differentiate individual cultures of a group can have a logical foundation or a permanent position unless it is founded upon quantitative data.

Notwithstanding the great accumulation of descriptions of bacterial species and varieties, it is difficult and in many cases impossible to identify any but the few which have been extensively

studied on account of their great importance as disease producers. In many cases, even these have varieties, subgroups, and atypical strains separated from the type by characters of doubtful significance. This is especially true of the colon group, a large family with very indefinite limits, radiating in all directions from the type organism, the *Bacillus coli communis*. Notwithstanding the fact that this organism is practically a normal inhabitant of the digestive tract and is found frequently in water, milk, and other material likely to become contaminated with feces, it is very difficult to establish the line of demarkation which separates *B. coli communis* from *B. lactis aerogenes* or others of its near relatives.

However imperfect may be our present methods of bacterial classification, there can be no question of the desirability of an accurate knowledge of the various important groups of bacteria whose existence is now recognized, in order that the limits of each group may be ascertained, the naturally related species established, their normal habitat worked out, and their various physiological functions studied in detail. Unfortunately, much of the description and naming of species has preceded the accurate study of the larger groups and the knowledge of the characters which for this particular group are significant and of value in determining the natural subdivisions. Bacteriologists have been much in the position of a biologist who casts a line in new waters, catches one fish, and uses this single specimen as the basis of a description of a new species. He may by chance have the type of a new species, or it may be an individual dwarfed by unfavorable conditions or changed by an unusual habitat. The bacteriologist who fishes a single culture from the unknown waters of the microscopic world has less chance of securing a type specimen, because he is working with organisms whose simple structure allows them to adapt themselves readily to new conditions and the number of varieties is correspondingly great.

The group in question, that usually designated as the colon group, is an excellent example, especially in its relation to water and milk, of the great need of exact work on an entire group. We have various names used to denote different members of the group, but the characters of each are by no means definite, and there is lit tle

evidence that the differentiation follows natural lines; we have tests drawn so closely that, presumably, they differentiate between strains of recent and remote fecal origin, but the tests are based on reactions of admittedly doubtful stability.

A study of the gas-forming bacteria of milk is essentially a study of this group. We have attempted to determine in some measure the significant characters which may be used in determining the natural limits of the group and the subdivisions which have arisen by descent from a common ancestor. We have given especial attention to the gas-producing function and its variations when determined by exact methods. This has been supplemented by determinations of the ability to ferment various carbohydrates, alcohols, and glucosides, the liquefaction of gelatin, the production of indol, and the reduction of neutral red and of nitrates. Finally, the results of these observations have been brought together and their relations to each other studied in an attempt to establish some, at least, of the natural families which it is believed must exist in a group of this size.

RESULTS OBTAINED.

Cultures studied.—The cultures used in this work were practically all obtained from milk or milk products, such as cheese and ice cream. Geographically they represented a wide distribution, including cultures obtained from Albert Lea, Minnesota; Madison, Wisconsin; Chicago, Ithaca, Boston, New York City, Baltimore, and Washington. The collection also contained a few cultures of established identity which were obtained for comparison. These included:

B. coli communis (*em*), a very old culture obtained from Professor C.-E. A. Winslow's collection in the American Museum of Natural History and coming originally through Dr. D. D. Jackson from the Kral collection.

B. aerogenes (*el*) from the same source.

B. coli communis (*fg*) from the Hygienic Laboratory of the U.S. Public Health Service.

B. coli communis (*fj*) from the laboratory of the University of Wisconsin Agricultural Experiment Station.

Bacillus of dysentery (Bitular) (*ev*) from the Biochemic Division of this bureau.

B. cholera suis (*ew*) isolated from a guinea-pig in the Biochemic Division of this bureau.

B. phytophytorus (*fp*), a very old culture coming originally from Apple and obtained from the laboratory of Plant Pathology of the Bureau of Plant Industry.

The usual procedure in isolating gas-forming cultures from milk was to plate the milk on lactose agar, incubate at 30°, and inoculate a large number of the resulting colonies in dextrose broth tubes with inverted inner tubes. All of these that showed

gas were replated and agar slope cultures made from the colonies. Usually only 2 or 3 cultures were made from one sample.

CHARACTERS STUDIED.

The cell.—These cultures showed so little variation in morphology that no attempt was made to use this character in differentiation. With possibly a few exceptions they all agreed with the short thick rod typical of the colon group. Spore formation was determined by staining and by exposing the culture to a temperature sufficiently high to destroy non-spore-forming cells. In nearly all cases these results were negative, but with a few cultures the results were uncertain. Nearly all of the cultures were gram negative, but as is well known this reaction is subject to some variation, and not infrequently the preparation showed both positive and negative cells.

Motility was not determined as it was believed that this character is too subject to variation owing to slight changes in the conditions. Burri and the earlier workers on this group placed much dependence on motility but in recent years it has been generally disregarded. Burk found it very difficult to determine motility with any certainty. Howe concluded that motility is not significant in the colon group. Jaffe says that motility is of no value in separating varieties of the group. Savage thinks that all true coli are motile but that when freshly isolated the motility may fail. Motility when observed may be additional evidence to assist in placing the culture but its failure is merely negative evidence.

Indol production.—While the production of indol is usually considered as one of the distinguishing characters of the colon group, it is recognized that it is subject to variation, and a negative test should not necessarily prevent a culture from being classed as *B. coli*. The Committee on Standard Methods of Water Analysis of the American Public Health Association makes the statement that indol production and nitrate reduction are variable and in a later report indol production is not included in the characters of the colon group altho it is used in the differentiation of some of the varieties. The indol test was made in the usual way on cultures incubated 7 days at 30° C. The results are given in Table 2.

Reduction of nitrates.—What has been said of the formation of indol is true also of the reduction of nitrates. The test is considered inconstant and of doubtful diagnostic significance.

This test was applied to cultures grown at 30° C. for 7 days in the following medium: peptone, 1.0 gm.; potassium nitrate, 0.2 gm.; water distilled, 1,000 c.c. The results are tabulated in Table 2.

Reduction of neutral red.—This test was found to be of some value in the differentiation of the lactic acid bacteria. Its value for other groups is doubtful, but all cultures were tested and the results are given in Table 2. To 1,000 c.c. of neutral broth were added 5 gm. of dextrose and 10 c.c. of 0.5 per cent solution of Grübler's neutral red. The neutral broth was made as follows: beef extract, 4 gm; peptone, 10 gm. and water, 1,000 c.c.

The tubes were examined after 7 days' incubation at 30° C. in an anaerobic jar from which the oxygen was absorbed with pyrogalic acid.

Liquefaction of gelatin.—The growth on gelatin and the liquefaction of the medium has always been considered of the greatest importance in differentiating bacteria, but in recent years there has been a growing tendency to attach less significance to the character of the growth or nature of the liquefaction and to depend on the fact of

liquefaction or non-liquefaction for identification, notwithstanding the generally recognized fact that this character is subject to considerable variation. In our own cultures it has been observed that several cultures giving a distinct liquefaction on the first test failed entirely on the second, a few weeks or months later. Of course in these cases the possibility of contamination was not absolutely excluded but it is not unlikely that the change was an instance of loss of function. The liquefaction of the gelatin is the expression of the attempt by the cell to secure nitrogenous food by the excretion of a proteolytic enzyme. When soluble nitrogen is supplied in excess of the needs of the organism the enzyme is no longer useful and it is not surprising that it is lost after a few generations on artificial media. In the fermentation of sugars, on the other hand, we have a function more closely associated with the protoplasm of the cell and probably with the production of energy essential to the activity of the cell and therefore less likely to variation.

We have used the method first suggested by Clark and Gage, and used by the Winslows in their work on the coccaceae and by us in the work on the lactic acid bacteria. This consists in inoculating the surface of a gelatin tube with a few drops of fluid culture, marking the top of the gelatin on a strip of paper pasted on opposite sides of the tube, and measuring the liquefaction after 30 days' incubation at 20° C. The results are given in Table 1.

TABLE 1.

LIQUEFACTION OF GELATIN.

Total Cultures	0 mm.	0–5 mm.	6–10 mm.	11–15 mm.	16–20 mm.	21–25 mm.	26–30 mm.	31–35 mm.	36–40 mm.	41–45 mm.	46–50 mm.	Over 50 mm.
122	109	1	0	0	1	4	3	0	1	0	1	2

These results will be discussed under another head.

Fermentation of carbohydrates.—The distinguishing characteristic of the gas-forming bacteria is their ability to form gas, acids, and other by-products from various carbohydrates, alcohols, and glucosides, and it is probable that in these reactions is found the most substantial basis for subdivisions of the group. The value of any reaction for this purpose depends (1) on its usefulness in showing lines of natural relationships, and (2) on its stability. It may be assumed that a character showing natural relationship would be stable since stability comes through repetition in many generations. There is, or has been, considerable difference of opinion in regard to the stability of the various manifestations of fermentative ability in bacteria. Not a few writers have asserted that the physiological reactions in general and the fermentation of sugars in particular are too variable to be used for purposes of classification. Burri found that old colonies frequently developed cells capable of producing gas from sugars not fermented by younger colonies. Gas formation was determined by shake agar cultures. Revis believes that physiological properties may be lost or acquired under action of competition and that a variation may become suddenly fixed. He found that when a typical *B. coli* was grown in a broth containing malachite green it gradually lost the power of forming gas altho it grew luxuriantly and typically on solid media. This new variety seemed to be permanent. Penfold in a series of papers shows the possibility of bacterial mutations produced partly under the influence of chemicals added to the media and partly under normal conditions through the

development of papillae on agar colonies. These new varieties which usually were non-fermenters were said to be permanent. On the other hand, Abbott, who was able to produce variations in *Sta. pyogenes aureus* by exposing repeated generations to various chemicals, found that this variation was in intensity of reactions rather than in the gain or loss of a function and that the sugar-splitting ability was not changed. Bergey using similar methods with *B. coli* was unable to obtain any mutations altho there was some evidence of alteration in some of the immunity reactions. Berry and Banzhof attempted to obtain by selection races of diphtheria bacilli with divergent powers of acid production and found that the strains instead of diverging tended to approach each other. Similar results were obtained by Buchanan and Truax working with streptococci. Revis, who produced atypical varieties by exposing cultures to malachite green, found that in cultures of *B. coli* held several months in sterile soil and in synthetic media there was no loss of any physiological function altho there was some variation in the intensity of the reaction. MacConkey held *B. coli* in water 358 days without change in its characters. MacConkey also gives the results of physiological tests on 15 cultures of *B. typhosus* from different sources including one that had been 16 years on artificial media. All of these cultures gave identical reactions. Similar results were obtained by Harding working with *Ps. campestris*.

The value of much of the work bearing on variations in bacteria, especially variations in the gas-forming function, is diminished by the inexact methods of measuring the reaction which, as will be shown in this paper, may lead to erroneous conclusions. It should also be remembered that, from the standpoint of systematic bacteriology, the important consideration is not the variations which may be formed by artificial conditions but the variations that occur in nature. If the cultural characteristics of a type organism are found originally by inexact methods, may we with reason say that slight variation from these characteristics is a true indication of variation in the physiological power of the organism? Conversely, if closer scrutiny reveals frequent variation among the cultural characteristics of the same organism, what hope is there of ever establishing tests sufficiently constant to be of diagnostic value?

In this vicious circle the larger question of natural selection and mutation are lost. It would therefore be in a certain sense a test both of the constancy of a particular medium and of a particular culture if it could be demonstrated that with the medium and culture in question the same products were reproducible *quantitatively*.

In dealing with certain questions, quantitative data alone will advance the science of bacteriology just as quantitative data alone have furnished the solution of problems in other sciences after qualitative experiments had plunged the subject into confusion.

Acid formation.—In studies of the bacteria of the colon group acid formation has usually been subordinated to gas formation as a measure of the fermentation of sugars and other test substances. Winslow in his work on the coccaceae has shown the value of the amount of acid formed not only because it gives exact results but also because in many cases varieties may be separated by the relative amounts of acid produced under given conditions. Some bacteria may produce sufficient acid in the absence of a fermentable sugar to affect the reaction of the broth sufficiently to change the color of an indicator and thus give misleading results. This condition is avoided by titration, which allows a distinction between the slight acidity that may come from any one of a variety of by-products and the marked acidity that usually comes from the fermentation of a carbohydrate.

TABLE 2

GENERAL CHARACTERISTICS OF ALL CULTURES.

CULTURE	MORPHOLOGY	SPORES	GRAM STAIN	MM. GELATIN LIQUEFIED IN 30 DAYS AT 20°	INDOL	NITRATES REDUCED	NEUTRAL RED REDUCED	PERCENTAGE LACTIC ACID FROM												
								Dextrose	Levulose	Galactose	Adonite	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Salicin	Dulcite
a	sr	−	−	0	−	+	+	.216	.000	.193	.000	.000	.324	.234	.000	.000	.270	.198	.000	.000
b	sr	−	−	24	−	+	+	.265	.207	.319	.000	.400	.198	.468	.099	.000	.234	.310	.468	.000
c	sr	−	−	28	−	+	+	.356	.144	.270	.000	.463	.207	.373	.108	.000	.333	.252	.441	.000
f	sr	−	−	23	−	+	+	.428	.477	.337	.000	.490	.333	.432	.000	.000	.378	.283	.495	.000
h	sr	−	#	23	−	+	+	.342	.468	.351	.000	.441	.072	.432	.126	.000	.360	.364	.459	.000
m	sr	−	−	0	−	+	+	.252	.288	.234	.021	.306	.324	.346	.162	.000	.369	.369	.531	.000
n	sr	−	+	0	−	+	+	.225	.090	.342	.423	.369	.270	.342	.189	.000	.216	.369	.531	.288
o	sr	−	−	0	−	+	−	.275	.378	.319	.549	.400	.373	.437	.477	.000	.360	.117	.549	.369
p	sr	−	−	0	−	+	+	.468	.513	.337	.000	.000	.342	.000	.000	.000	.414	.260	.504	.000
s	sr	−	−	0	−	+	+	.297	.468	.335	.423	.387	.310	.237	.468	.054	.342	.265	.531	.000
u	sr	−	−	0	−	+	+	.360	.540	.180	.297	.468	.153	.318	.342	.000	.333	.265	.567	.000
w	sr	−	#	0	−	+	+	.126	.144	.153	.000	.252	.207	.387	.000	.000	.306	.144	.468	.000
ag	sr	−	#	0	−	+	+	.108	.198	.126	.423	.306	.279	.252	.133	.000	.288	.117	.369	.000
aj	sr	−	−	0	−	+	+	.153	.450	.108	.153	.207	.270	.216	.333	.000	.288	.135	.477	.000
ak	sr	−	−	0	−	+	+	.576	.558	.297	.000	.387	.333	.504	.000	.000	.405	.117	.369	.450
al	sr	−	−	0	−	+	+	.540	.531	.441	.000	.396	.243	.531	.000	.000	.396	.135	.495	.522
ao	c	−	#	0	−	+	+	.000	.153	.270	.504	.216	.207	.117	.000	.000	.252	.036	.540	.000
ap	sr	−	−	0	−	+	+	.513	.108	.270	.504	.369	.189	.405	.324	.000	.126	.072	.540	.126
aq	sr	−	−	0	−	+	+	.495	.540	.315	.000	.333	.351	.000	.000	.000	.369	.261	.297	.540
ar	sr	−	−	0	−	+	+	.486	.531	.243	.009	.000	.189	.000	.000	.045	.018	.000	.063	.009
aw	sr	−	−	0	−	+	+	.576	.441	.351	.000	.000	.378	.000	.000	.000	.387	.288	.036	.000
ax	sr	−	−	0	−	+	+	.576	.549	.351	.000	.000	.378	.000	.000	.000	.360	.297	.378	.549
ay	c	−	#	0	−	+	+	.495	.414	.333	.000	.000	.324	.000	.000	.000	.369	.126	.486	.423
az	sr	−	−	0	−	+	+	.540	.585	.351	.000	.000	.360	.000	.000	.000	.405	.144	.369	.000
ba	sr	−	−	0	−	+	+	.558	.549	.378	.000	.000	.360	.000	.000	.000	.369	.207	.351	.567
bb	sr	−	−	0	−	+	+	.549	.531	.414	.000	.000	.216	.000	.000	.000	.360	.234	.297	.513
bc	sr	−	−	0	−	+	+	.621	.549	.216	.441	.000	.297	.243	.000	.000	.423	.108	.540	.000
bd	sr	−	−	0	−	+	+	.549	.423	.324	.000	.000	.351	.000	.000	.000	.432	.252	.000	.000
be	sr	−	−	0	−	+	+	.558	.360	.396	.000	.000	.369	.225	.000	.000	.414	.234	.459	.000
bf	lr	+	−	0	−	+	+	.531	.549	.351	.000	.000	.369	.000	.000	.000	.360	.306	.387	.387
bg	sr	−	−	0	−	+	+	.576	.540	.198	.000	.000	.387	.000	.000	.000	.396	.171	.558	.495
bh	sr	−	−	0	−	+	+	.621	.576	.315	.000	.000	.315	.000	.000	.000	.459	.099	.332	.340
bi	sr	−	−	0	−	+	+	.540	.495	.342	.000	.000	.378	.000	.000	.000	.405	.288	.000	.000
bj	sr	−	−	55	−	+	+	.135	.144	.216	.000	.351	.036	.063	.513	.000	.216	.234	.558	.000

bk........	sr	−	−	29	+	+	+	.144	.297	.279	.000	.319	.301	.018	.144	.000	.396	.225	.531	.000
bl........	sr	−	−	0	+	+	+	.126	.189	.126	.702	.387	.369	.387	.270	.000	.342	.126	.342	.000
bm........	sr	−	+	0	−	+	+	.549	.540	.432	.000	.000	.387	.009	.000	.000	.450	.216	.135	.477
bn........	c	−	+	27	−	+	−	.387	.585	.180	.000	.450	.180	.000	.000	.036	.108	.324	.567	.027
bo........	sr	−	+	0	+	+	+	.126	.135	.189	.387	.225	.369	.270	.288	.000	.270	.135	.459	.252
bp........	sr	−	−	0	+	+	+	.612	.549	.432	.000	.000	.351	.000	.063	.045	.414	.135	.054	.000
bq........	c	−	±	0	−	+	+	.126	.126	.252	.306	.477	.135	.252	.261	.000	.144	.072	.450	.000
br........	sr	−	−	0	+	+	+	.072	.270	.144	.549	.234	.288	.252	.288	.063	.333	.054	.495	.000
bs........	sr	−	−	0	+	+	+	.090	.225	.360	.459	.153	.261	.117	.234	.414	.270	.126	.396	.000
bt........	sr	+	−	0	+	+	+	.522	.558	.450	.000	.540	.333	.585	.000	.000	.378	.252	.477	.009
bu........	sr	+	−	0	+	+	+	.135	.117	.261	.000	.315	.126	.315	.207	.000	.423	.027	.504	.252
bv........	sr	−	−	0	+	+	+	.441	.477	.261	.288	.427	.324	.171	.198	.000	.360	.099	.585	.000
bw........	sr	−	−	0	+	+	+	.117	.216	.288	.576	.369	.234	.207	.243	.000	.180	.108	.540	.000
by........	sr	−	−	0	−	+	+	.189	.108	.108	.612	.333	.324	.270	.180	.000	.315	.099	.495	.000
bz........	sr	−	±	0	−	+	+	.441	.432	.189	.027	.432	.324	.279	.234	.000	.324	.162	.477	.000
ca........	sr	+	−	0	+	+	+	.135	.270	.135	.495	.270	.252	.162	.198	.000	.234	.099	.414	.234
cb........	sr	−	−	0	+	+	+	.180	.225	.243	.473	.234	.333	.117	.225	.000	.162	.090	.486	.198
cc........	sr	−	−	0	+	+	+	.153	.126	.198	.594	.306	.207	.171	.198	.000	.225	.126	.504	.198
ce........	sr	−	−	38	−	+	−	.423	.171	.216	.000	.414	.099	.486	.432	.000	.315	.270	.459	.000
cf........	sr	+	−	0	+	+	+	.396	.486	.324	.441	.000	.270	.144	.000	.000	.378	.198	.540	.000
cg........	sr	−	−	0	+	+	+	.252	.153	.135	.513	.306	.369	.333	.396	.576	.324	.036	.504	.279
ch........	sr	−	−	0	+	+	+	.270	.225	.261	.612	.279	.279	.216	.243	.000	.225	.090	.459	.198
ci........	sr	−	−	0	+	+	+	.054	.099	.279	.000	.000	.720	.081	.000	.000	.279	.063	.000	.000
cj........	sr	−	−	46	+	−	+	.351	.162	.216	.000	.378	.405	.364	.036	.000	.315	.162	.396	.000
ck........	sr	−	±	22	+	+	+	.171	.135	.234	.477	.423	.234	.252	.270	.000	.324	.153	.540	.000
cl........	sr	−	−	0	+	+	+	.171	.225	.198	.531	.288	.315	.216	.279	.000	.252	.117	.486	.000
cm........	sr	−	−	0	−	+	+	.144	.540	.126	.000	.315	.270	.153	.099	.000	.243	.117	.576	.000
cn........	sr	−	−	0	+	+	+	.117	.225	.135	.000	.315	.225	.603	.081	.018	.171	.162	.585	.018
cq........	sr	−	−	0	−	+	+	.126	.180	.126	.000	.198	.297	.387	.000	.000	.090	.171	.522	.000
cr........	sr	−	+	0	−	−	+	.180	.162	.270	.000	.180	.288	.459	.000	.000	.108	.154	.450	.000
cs........	sr	−	−	0	+	+	+	.189	.126	.108	.000	.243	.207	.198	.000	.000	.126	.117	.432	.000
ct........	sr	−	−	0	+	−	+	.189	.270	.153	.603	.378	.315	.306	.117	.000	.126	.153	.441	.252
cu........	sr	−	−	0	+	+	+	.117	.126	.117	.459	.288	.180	.270	.288	.000	.252	.072	.477	.000
cv........	sr	−	−	0	−	+	+	.117	.126	.279	.531	.279	.063	.288	.270	.000	.270	.180	.387	.297
cw........	r	−	±	0	−	+	−	.495	.468	.405	.000	.000	.324	.000	.000	.000	.369	.225	.054	.000
cx........	r	−	−	0	+	+	+	.531	.126	.351	.000	.000	.333	.090	.000	.000	.360	.135	.504	.477
cy........	sr	−	−	0	+	+	+	.162	.495	.045	.000	.099	.144	.234	.000	.000	.126	.135	.477	.000
cz........	sr	−	−	0	+	+	+	.261	.414	.261	.423	.423	.225	.369	.126	.000	.324	.117	.198	.000
da........	sr	−	+	0	+	+	+	.522	.405	.180	.000	.000	.369	.000	.000	.000	.396	.396	.468	.396
db........	sr	−	±	0	+	+	+	.558	.522	.360	.000	.000	.378	.000	.000	.000	.387	.162	.432	.468
dc........	sr	−	−	0	+	+	+	.558	.522	.369	.000	.387	.234	.648	.000	.000	.396	.297	.306	.540
dd........	sr	+	−	0	+	+	+	.558	.567	.360	.000	.342	.306	.675	.000	.000	.450	.306	.423	.488
de........	sr	−	+	0	−	+	+	.252	.261	.144	.423	.387	.351	.270	.243	.036	.225	.099	.432	.000
df........	sr	−	−	0	+	+	+	.540	.576	.270	.000	.153	.342	.585	.000	.000	.369	.288	.288	.000
dg........	sr	−	−	0	+	+	+	.603	.522	.144	.000	.315	.297	.000	.000	.000	.405	.162	.027	.414
dh........	r	−	−	0	+	+	+	.621	.144	.369	.495	.387	.414	.000	.000	.000	.360	.360	.459	.000
di........	sr	−	−	0	+	+	+	.531	.180	.414	.216	.000	.288	.000	.000	.000	.432	.288	.486	.000
dj........	sr	−	−	0	+	+	+	.081	.189	.135	.594	.216	.126	.261	.027	.000	.198	.189	.171	.000

TABLE 2—*Continued.*

Culture	Morphology	Spores	Gram Stain	Mm. Gelatin Liquefied in 30 Days at 20°	Indol	Nitrates Reduced	Neutral Red Reduced	Percentage Lactic Acid From										
								Dextrose	Levulose	Galactose	Adonite	Saccharose	Lactose	Raffinose	Starch	Inulin	Mannite	Glycerin	Salicin	Dulcite
dk	lr	−	−	o	+	+	+	.567	.549	.306	.000	.342	.369	.657	.000	.000	.423	.126	.297	.378
dl	r	−	−	o	+	+	+	.594	.513	.297	.000	.369	.351	.666	.000	.000	.387	.270	.477	.540
dm	sr	−	−	o	−	+	+	.117	.522	.225	.000	.306	.333	.216	.288	.000	.216	.099	.423	.135
dn	sr	−	−	o	+	+	+	.081	.108	.117	.000	.288	.306	.207	.045	.000	.315	.207	.549	.000
do	sr	−	−	o	+	+	+	.562	.540	.408	.000	.522	.387	.588	.000	.000	.396	.144	.468	.513
dp	sr	−	−	o	+	+	+	.414	.099	.162	.405	.378	.261	.234	.351	.000	.387	.045	.531	.090
dq	sr	−	−	o	+	+	+	.603	.171	.306	.000	.000	.306	.000	.000	.000	.423	.342	.045	.000
dr	r	−	−	o	+	+	+	.549	.522	.297	.009	.000	.309	.000	.000	.000	.443	.261	.387	.000
ds	sr	−	−	o	+	+	+	.684	.810	.253	....	.594	.522	.639	.072	.000	.432	.135	.639	.585
dt	sr	−	−	o	+	+	+	.558	.018	.378	.000	.468	.378	.000	.000	.000	.423	.360	.369	.000
du	r	−	−	o	+	+	+	.531	.594	.369	.000	.000	.378	.423	.000	.000	.441	.378	.414	.522
dw	r	−	−	o	+	+	+	.513	.540	.333	.000	.450	.432	.000	.000	.000	.450	.270	.423	.000
dv	sr	−	−	o	+	+	+	.223	.144	.144	.423	.414	.288	.333	.000	.000	.216	.085	.486	.000
dz	sr	−	−	o	+	+	+	.090	.225	.162	.000	.153	.288	.113	.000	.000	.270	.198	.558	.459
ea	sr	−	−	o	+	+	+	.567	.540	.369	.000	.000	.306	.123	.000	.000	.414	.108	.315	.000
ec	sr	−	−	o	+	+	+	.567	.549	.351	.405	.657	.207	.000	.000	.000	.405	.207	.468	.000
ed	r	−	−	o	+	+	+	.558	.531	.324	.405	.000	.324	.000	.000	.000	.351	.117	.459	.000
ee	sr	−	−	o	+	+	+	.551	.567	.351	.000	.000	.360	.508	.000	.000	.369	.207	.405	.567
ef	sr	−	−	o	+	+	+	.513	.342	.342	.000	.477	.387	.144	.000	.000	.396	.252	.009	.342
eh	sr	−	−	o	+	+	+	.504	.387	.369	.000	.000	.270	.513	.027	.000	.387	.117	.009	.513
ej	sr	−	−	o	+	+	+	.504	.378	.300	.000	.396	.198	.027	.000	.000	.405	.117	.396	.414
ek	sr	−	−	o	+	+	+	.549	.576	.333	.018	.351	.279	.000	.000	.000	.378	.288	.081	.000
el	r	−	−	o	+	+	+	.504	.459	.225	.000	.261	.261	.540	.000	.000	.594	.162	.477	.558
em	sr	−	−	o	+	+	+	.441	.441	.324	.000	.000	.054	.000	.000	.000	.000	.000	.072	.009
en	sr	−	−	o	+	+	+	.541	.000	.324	.000	.351	.144	.207	.000	.540	.225	.009	.594	.207
eq	sr	−	−	5	+	+	+	.585	.531	.144	.432	.000	.315	.000	.000	.036	.603	.144	.459	.000
er	sr	−	−	o	+	+	+	.594	.558	.207	.459	.000	.342	.117	.000	.000	.567	.072	.018	.000
es	sr	−	−	o	+	+	+	.567	.549	.477	.000	.000	.333	.090	.000	.000	.531	.171	.351	.000
et	r	−	−	o	+	+	+	.126	.198	.261	.540	.270	.207	.333	.009	.000	.495	.153	.432	.225
eu	sr	−	−	o	−	+	+	.531	.531	.477	.000	.000	.342	.000	.000	.000	.532	.135	.306	.000
ev	sr	−	−	....	+	+	+	.126	.126	.162	.297	.108	.252	.315	.090	.000	.378	.162	.306	.000
ew	sr	−	−	o	+	+	+	.279	.441	.441	.000	.000	.000	.000	.027	.000	.657	.153	.414	.594
ey	r	−	−	o	−	+	+	.009	.045	.189	.000	.153	.234	.126	.117	.432	.477	.171	.054	.000
fa	sr	−	−	o	−	+	−	.360	.000	.000	....	.000	.261	.000	.000	.000	.549	.090	.459	.000
fb	sr	−	−	50	+	+	+	.522	.225	.414	.000	....	.261	.342	.000	.000	.549	.090	.459	.000
fc	sr	−	−	o	+	+	+	.126	.171	.117	.171	.261	.315	.243	.117	.000	.261	.126	.360	.000
fd	sr	−	−	o	+	+	+	.225	.252	.225	.000	.216	.117	.396	.018	.000	.324	.108	.324	.000
fe	sr	−	−	o	+	+	+	.189	.171	.261	.000	.216	.162	.396	.027	.000	.153	.135	.387	.000
ff	sr	−	−	o	+	+	+	.135	.243	.054	.000	.072	.279	.423	.036	.000	.297	.108	.387	.000
fg	sr	+	−	o	+	+	−	.225	.468	.450	.000	.144	.233	.324	.027	.000	.396	.270	.333	.324

We have used in our tests for fermentative ability the sugars, dextrose, levulose, galactose, adonite, saccharose, lactose, and raffinose, the polysaccharides starch, and inulin; the alcohols mannite, glycerin, the glucosid salicin and dulcite. These were used in a broth made as follows: beef extract, 0.4 per cent; peptone, 1 per cent; dibasic potassium phosphate, 0.5 per cent, and test substance, 1 per cent. The broth was brought to the neutral point before the addition of the potassium phosphate. The cultures were incubated at 30° C. for 7 days. Some objections may be made to the use of a temperature lower than that ordinarily used with the colon group and one which is below the optimum temperature of many of the cultures. This temperature was selected rather than the more usual one of 37° C., because while many of our cultures grew very slowly at the higher temperature, all grew readily at 30° C. Moreover, the difference in growth at these temperatures is only in rapidity and the maximum acidity, which is reached at either temperature in considerably less than 7 days, is approximately the same in both cases. Five cubic centimeters of the broth were titrated against twentieth normal sodium hydrate after the tubes had been held 15 or 20 min. in a steam bath to drive off as much as possible of the carbon dioxid. The results expressed as percentage of lactic acid are given in Table 2. In all cases the titer of a blank was subtracted from that of the culture.

It was observed that in many cases in which there was a comparatively high acidity in lactose and other sugars which are presumably fermented with some difficulty there was a low acidity and even a neutral or alkalin reaction in the broth containing dextrose, levulose, or galactose. This is due, not to the failure to ferment the sugar, but to the production by some varieties, after the sugar fermentation has ceased, of some substance with an alkalin reaction which in time may entirely overcome the earlier acidity of the culture. This phenomenon is frequently observed in lactose litmus plate cultures of colon organisms. This alkali formation evidently begins after the completion of the acid fermentation and is therefore more evident in a broth with an easily fermentable sugar like dextrose than with lactose or other of the more complex sugars. This property of alkali formation with the consequent tendency to uncontrolled variation reduces very materially the value of the titer of sugar broths for diagnostic purposes.

The fermentation of dextrose has been determined with every culture by the gas test and therefore this sugar has been considered positive even when the final reaction has given no indication of fermentation. Levulose, galactose, and salicin were in nearly every case fermented at about the same rate as dextrose and consequently were of little value in differentiating one culture from another.

Gas formation.—While we have used the ordinary methods for the foregoing cultural tests, in the study of gas production we have endeavored to find whatever value exact methods may reveal. Our investigations have been concerned with three points of deep interest: the value of exact methods in gas analysis for diagnosis, the constancy of the gas-producing powers of our cultures, and the mechanism of the gas production. Upon this last problem our first series of experiments are not yet complete. The constancy of the physiological characteristics of the bacteria with which we are dealing is a subject which requires not only a preliminary survey but time for the attainment of rigid conclusions, and we shall, therefore, discuss it only incidentally pending more extensive research. Upon the value of exact methods of gas analysis for diagnostic purposes we feel that the preliminary survey we have made has furnished results worthy of publication, and it is with this phase of our researches that we now have to deal.

The ordinary routine methods used in study of the gas production by bacteria are unsuited for quantitative work. The limitations of the Smith fermentation tube were pointed out by the originator and more recently elaborated by Keyes and by Burri and Düggeli.

The chief objections may be summarized as follows:

1. Owing to the solubility of carbon dioxid a large percentage of that product is retained by the medium, and, consequently, an analysis of the supernatant gas does not give a true indication of the volume of gas actually liberated in the fermentation, nor a true ratio between the carbon dioxid and other gases.

2. The medium is exposed to an atmosphere of high carbon dioxid tension in the closed arm and an atmosphere of low carbon dioxid tension over the open arm. Because of this and the high solubility of carbon dioxid in the medium separating the two atmospheres this gas diffuses into the open arm and is lost.

3. The closed arm is anaerobic, the open arm aerobic and the volume of medium exposed to each, as well as the volume of medium furnishing gas to the closed arm, is constantly changing.

One or more of these objections apply to almost all of the methods which are commonly used. The first criticism based upon the solubility of carbon dioxid is perhaps the most widely applicable.

It applies among others to the methods of Escherich, Hesse, Gärtner, Bennett and Pammel, Pakes and Jollyman, Schittenhelm and Schröter, Salus, Fuhrman, and finally that of Harden, Thompson, and Young if used without the corrections they mention. The U-tube of Dunbar was of course practically the same as the device introduced by Smith in 1890. Modifications of the Smith tube with little to recommend them but the ease with which the gas may be drawn for analyses are found in the devices of Ampola and Garino, Pennington and Küsel, Hofstädter, Silberger, Beijerinck and Minkmann, and McCrudden. In addition to these are manometric methods for estimating simply the total gas, as that of Söhle.

Not all of these were employed in studying the gases produced by *B. coli;* but, if we assemble some of the numerous analyses[1] of the gas produced by this extensively studied bacillus, and compare them with analyses made by exact methods, the extent of the error under consideration will become apparent.

To describe in detail each of the various methods employed would be tedious; and while it might be of value, the comparison can be done more thoroughly and justly if the reader has before him the original papers with all the details including the evidence that the bacterium under consideration is a true colon. It will serve the purpose if we simply sketch the results of a few of the many researches.

[1] In the analyses which are to be quoted as well as in those which we shall have to present we shall depart from the custom of expressing the ratio as H_2/CO_2. Instead we shall use the inverted ratio CO_2/H_2. The reason for so doing is twofold. In the first place, we have found in our own analyses that, almost without exception, the volume of carbon dioxid exceeds that of hydrogen. Consequently the ratio CO_2/H_2 is the more convenient one to handle. In the second place, this ratio is more easily converted into the fractional values used rather generally. Thus the ratios $\frac{CO_2}{H_2} = 2.33, 1.20, 1.00$, may easily be read as $\frac{H_2}{CO_2} = \frac{1}{2.33}, \frac{1}{1.20}, \frac{1}{1}$. Such expressions containing a uniform numerator are more easily intercompared, than the equivalent 3/7, 5/11, 1/1. Furthermore, the ratio CO_2/H_2 has priority to H_2/CO_2 in that it was used by Escherich in 1886. Attention should also be called to the use of the molecular formula of hydrogen, H_2. Various authors, including Escherich and Smith, employ merely the symbol H, a usage very confusing to a chemist, since the data are in terms of hydrogen volumes and should be properly designated as H_2.

Escherich is quoted by Scruel to the effect that the gas produced by *B. coli* is composed of hydrogen, carbon dioxid, and methane. Methane, however, seems to have been mentioned by Escherich only as a constituent of intestinal gas. Escherich found a ratio of CO_2/H_2 approximately equal to 1 for the gases produced by his *B. coli communis* when grown in the presence of $CaCO_3$. This ratio was also found by Chantemesse and Widal and rather widely quoted. But this was criticized by Dunbar on the basis that considerable carbon dioxid must have been liberated from the action of the acids formed upon the calcium carbonate present in the medium used by Chantemesse and Widal. The same criticism applies to Fremlin's experiment in which his medium contained 3 per cent soda.

In Harden's study of the fermentation of glucose by *B. coli* he used in his medium an excess of $CaCO_3$; but he made an attempt to correct not only for the carbon dioxid liberated from this by the acids formed, but also for carbon dioxid retained in solution. With these corrections his data furnished ratios approximating unity. His estimation of the carbon dioxid retained in solution was more or less inaccurate because he failed to consider that there were present all the materials for the carbamino reaction. Upon precipitating (Siegfried) the dissolved carbon dioxid with baryta water it is probable that some carbon dioxid remained in solution associated with the amino bodies and alkalin earths present. If so, the estimate of the CO_2 in solution was too low and the true ratio of CO_2/H_2 was probably greater than 1. Keyes calls attention to the fact that Harden's estimate of the CO_2 dissolved in one instance cannot be applied justly to other cases, since the partial pressure of CO_2 above the liquid varied in each case. To this we may add that reactions taking place in a medium kept neutral with $CaCO_3$ may be very different from those occurring with progressive changes in reaction.

That the ratio is CO_2/H_2 equals 1/1 for the gases *produced* by *B. coli* from dextrose seems, therefore, to have been the fortunate conclusion of several ill-considered investigations. It proved to be fortunate because of the claim of Scruel that the gases were liberated by the decomposition of the formic acid formed in the fermentation, a theory which became the inspiration of a number of profitable researches. These and the one-to-one ratio which Scruel's equation, $CH_2O_2=CO_2+H_2$, would demand of the gases produced by *B. coli* have been lost sight of to a large extent in the numerous investigations on the gas production of bacteria which were made for diagnostic purposes. In these investigations the starting-point was the classic work of Theobald Smith, and the methods were designed with the idea of obtaining results not of analytical accuracy but of comparative value.

With the fermentation tube which he introduced Smith obtained from the action of *B. coli* on dextrose $CO_2/H_2=\frac{1}{2}$. The results obtained by numerous investigators, among whom may be mentioned Pennington and Küsel, Bennet and Pammel, and Mendel, are essentially the same as those found by Smith, and are characterized by the excess of hydrogen over carbon dioxid, owing to the fact that the methods made no provision for the collection of the carbon dioxid held in solution.

Smith's analyses show $CO_2=31.5$–37.2 per cent, $H_2=62.8$–68.5 per cent. Lembke concluded that such results were not reproducible. He found:

6	times	CO_2/H_2	$=1/7$
2	"	"	$=1/6$
4	"	"	$=1/5$
4	"	"	$=1/4$
4	"	"	$=1/2$

Wolffin found $CO_2=22$ per cent, $H_2=75.6$ per cent, $N=2.1$ per cent. Stamm contended that 72 hours' growth was necessary to obtain consistent results and published an analysis showing $CO_2=31.37$, $H_2=58.8$ per cent. Without strict anaerobiosis Pennington and Küsel found an average of 2.09 per cent methane,[1] yet with strict anaerobiosis they could discover no methane in the gas, but found an average of 32.4 per cent CO_2; 64.2 per cent H_2; 3.4 per cent N_2. Bennet and Pammel found 32 per cent CO_2; 68.0 per cent H_2; Fuhrmann found 30.84 per cent CO_2, 62.86 per cent H_2, 6.5 per cent residue; and Pammel and Pammel, 24 per cent CO_2, 75 per cent H_2.

Altho the variation in individual analysis is rather wide, essentially the same results are mentioned by numerous other authors who give merely the ratio $CO_2/H_2=\frac{1}{2}$.

Among these analyses there is a greater degree of uniformity than Keyes seems inclined to admit. Indeed the comparisons in Keyes's table are not at all happily made, for there are included with the comparable results of Smith, and Pammel and Pammel those of Grimbert, whose medium contained nitrate; the analysis of Schittenhelm and Schroter,[2] who were working with nucleic acids, and the work of Harden who placed his analysis beyond comparison with the others by correcting for dissolved CO_2.

Nevertheless, it takes but a glance at the numerous published analyses of the gas produced by *B. coli* to make one realize that such agreement as exists is attained by the selection of typical analyses, and that Keyes's chief contention, that there are hopelessly wide variations due to inaccuracies of method, is all too true. Perhaps no better illustration is to be found than in the statistical treatment which Longley and Baton have made of a large number of analyses by themselves and others. The statistics presented by Longley and Baton, which are typical of those in other investigations carried out under similar conditions and which indicate "that the quantity of gas formed in confirmatory fermentation tubes varies within wide limits," makes one sympathize with their statement that, "generally speaking, all the evidence we have seems to indicate that there is no determinative significance attached to the quantities of gas and carbon dioxid in the confirmatory or subculture fermentation tube." Even when these authors attempted to overcome the error due to absorption of carbon dioxid in the medium by first saturating the medium with this gas, they obtained, instead of the typical fermentation tube ratio for *B. coli* of $CO_2/H_2=\frac{1}{2}$, the ratio 3/1, a value very far from that obtained in the more exact investigations, which we have to record, or which Harden or Keyes have presented.

The conclusions of Longley and Baton, as well as those of Howe, to the effect that the gas volume and ratio are in a quantitative sense not reliable for diagnostic purposes, is a conclusion which, we shall see, our results strikingly disprove.

Determination of gas formation by our cultures, made by the usual methods, gave very discordant results. Were we to seek correlations from these data we should encounter the same difficulties met by Longley and Baton, and by Howe.

In view of the difficulties which many workers seem to encounter in obtaining consistent data with the Smith fermentation tube, it is perhaps not out of place for us to suggest that workers must agree upon a more uniform procedure. Nothing is

[1] We should be grateful if told the authority for the statement recurring in textbooks that Klein found the combustible gas to be methane.

[2] Compare Oppenheimer, *Zeit. f. Physiol. Chem.*, 1904, 41, p. 3.

to be gained by the addition of new methods like that of Burri and Düggel , which is similar to that of Seiffert and which has nothing to commend it in the way of accuracy. Nor is there anything to be gained by cumbersome apparatus such as that of Robin or that of Epstein, which retain the gravest fault of the Smith tube.

The chief improvement must involve some means of recovering the gas held in solution by the medium. Two obvious methods for collecting this dissolved gas are: (1) to sweep it out with a neutral gas aided by high temperature if necessary and collect it in a suitable train of absorbents; or (2) to pump it out and analyze it by the ordinary gasometric procedure.

The first method has been elaborated by Stocklasa in his study of nitrogen assimilation. Duchácêk used it in studying the CO_2 output of the typhoid and colon bacteria. The difficulties attending a very accurate determination of hydrogen in such a procedure combined with the necessity for employing rather large quantities of medium make this method objectionable for the study of hydrogen-producing bacteria or for investigations involving the use of expensive material.

For the collection of gas produced by bacteria the mercury pump has been used by Godlewskiego in the study of nitrifying bacteria; by Oppenheimer in some work on intestinal gases; and by Keyes for collecting the gases of *B. coli*. Keyes's paper and his reasons for replacing the Smith tube have already been referred to.

The method we have used is essentially that of Keyes. Several modifications, however, have been made, which we have found better adapted to our work, and which we will describe in detail.

DESCRIPTION OF THE APPARATUS.

The culture bulb used by Keyes we have replaced by a less expensive and more trustworthy form. This is shown in Figs. 1 and 2. It bears a resemblance to one of Roux's Pasteur tubes. The side arm serves for the introduction of the medium and for its inoculation, after which it is sealed off. The other constricted tube is used for the exhaustion of air, after which it also is sealed off. During the period of incubation the bulb is infallibly sealed, and may be left indefinitely without any of the suspicions which stopcocks arouse. After use these bulbs may easily be repaired by anyone having ordinary skill in glass-blowing.

For the first exhaustion we have used the familiar Boltwood mercury pump.

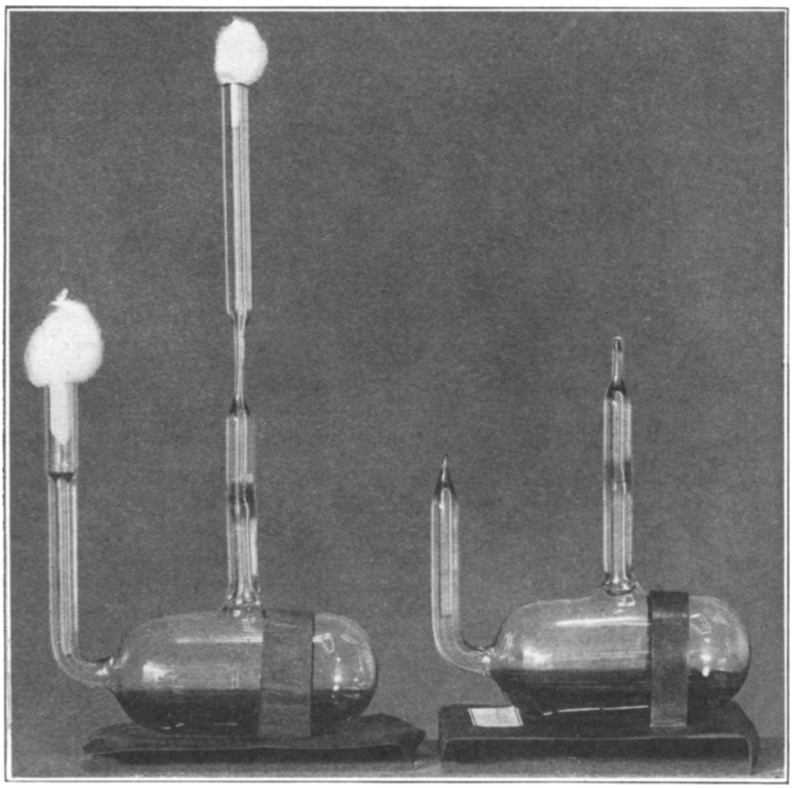
For the collection of the gas we have used the Antropoff modification of the Toepler pump, illustrated in Fig. 3. The essential modification is the inclination of the chamber *A* which prevents in a very effective way the disastrous pounding of the mercury as the gas bubbles through it. By inclining the chamber the gas passes in between the mercury and the upper wall with remarkable smoothness so that a full stroke may be taken at any time without danger of smashing the pump. Two of these pumps which we made in our own laboratory have worked so smoothly that this opportunity is taken to add our recommendation to the many which Dr. Antropoff's simple modification has received.

During the preliminary exhaustion of the bulb with the Boltwood pump the state of the vacuum was estimated by the nature of an electric discharge through a Plücker tube. The evacuation was continued, until, with the connecting stopcock open, the indication of a high vacuum heard in the characteristic sharp click of the mercury in the fall tube was confirmed by the absence of the discharge in the Plücker tube. It is

estimated that the pressure of the gas exclusive of water vapor left in the bulb was well below 0.005 mm.

For estimating the vacuum during the collection of the gas with the Antropoff pump a McLeod gauge was used.

To connect the bulb with the Boltwood pump the straight upright tube of the bulb is slipped through a perforated rubber nipple such as is used for nursing bottles, and is pushed into a short piece of rubber tubing, tied to the inlet of the pump, until



FIGS. 1 AND 2.—Fermentation bulbs.

the ends of the glass tubes are squarely met. The rubber tube is then securely tied with shoemaker's waxed thread. Now the rubber nipple is brought up till it covers the connection and it is then filled with mercury. This provides a mercury-sealed joint which is both simple and effective. This same form of seal is used in making the connection with the Antropoff pump but the inlet of this is modified. To collect the gas the sealed tip of the bulb (Fig. 2) has to be broken, and in order that this may be accomplished the inlet was so blown that it might receive the taper of the seal as shown in detail, Fig. 4. At the point *e* the tube was collapsed to an elliptical cross-section,

so that the tip of the seal when broken off cannot jam; *f* is a piece of stout rubber tubing tied with waxed thread, and *g* a rubber nipple filled with mercury.

Above the connection in each pump is a mercury-sealed stopcock. By opening this only occasionally there is prevented a continuous distillation of water from the medium into the drying agent.

The drying tubes of both pumps are for sulfuric acid. While sulfuric acid, if given time and proper circulation, is quite as good a drying agent as phosphorus pentoxid, it is of course not so efficient for rapid drying. The small difference, however, is compensated for by the greater ease with which sulfuric acid may be renewed, a factor which is of considerable importance when a large number of pumpings are to be made, and when none of these need to be the extremely high vacuum necessary in other classes of work.

The form of drying tubes shown in Fig. 3 has proved very satisfactory. The acid is poured in at *a* until it fills the tube some distance above the horizontal body *b*. Upon draining the acid out through *e* the walls are left "moistened." It is only drained, however, until there is a layer half filling the body, and there is left a clear space above for the flow of gas. The stopcocks *a*, *e*, and *f* are mercury-sealed, and by leaving a few drops of acid in *a* and keeping the tube *c* filled by trapping the acid with a beaker, as shown in the figure, the cocks are made perfectly gas-tight.

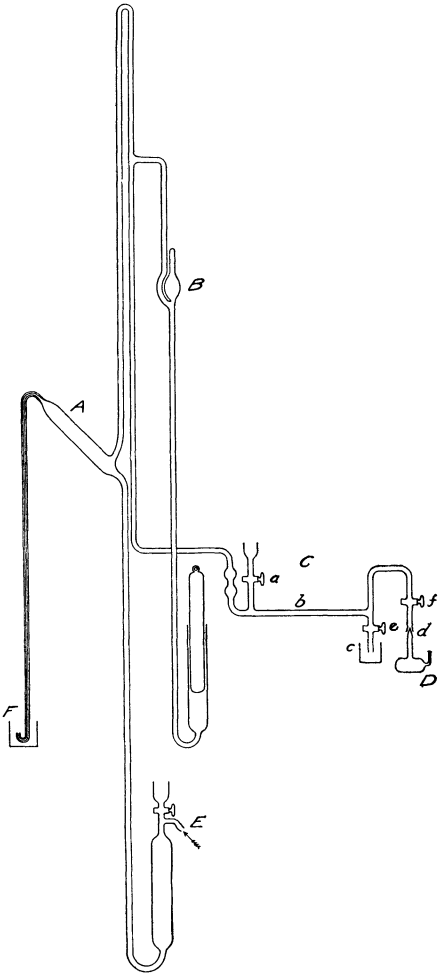


FIG. 3.—Vacuum pump and connections.

The initial action of the acid on the stopcock lubricant is troublesome but this soon disappears, and if care be taken to avoid jamming the cocks in the effort to make them snug the lubricant will last for months. This form of drying tube has proved to be capable of some very nice adjustments. By providing the bulbs as shown, the tube may be filled with the acid sufficiently to force the gas to bubble through it, thereby giving it additional efficiency in a preliminary drying of the pump. During an evacuation, of course, a free passage must be allowed for the gas, but the leveling of the tube may be easily adjusted, so that if too rapid a current of gas is accidentally allowed to enter, it will cause a wave of acid to be quickly formed. This, closing across the tube, requires the gas to bubble through it. Then, when the current of gas has subsided, the acid returns to its normal level, leaving

a clear passage above. It is believed that more efficient drying is procured by having the entering gas impinge directly upon the layer of acid. If during the evacuation of a large body of medium it should be thought desirable to renew the acid, the pump may be stopped and brought to atmospheric pressure and the acid renewed. The pump is then re-evacuated without the vacuum already created in the vessel being exhausted, provided the cock between the vessel and the pump is kept closed.

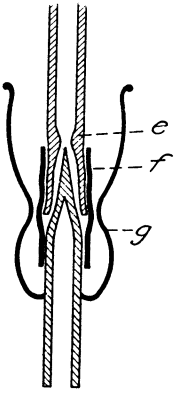


FIG. 4.—Method of joining bulb to pump.

The detailed procedure is as follows: With the open arms of the fermentation bulb plugged with cotton the clean dry bulb is sterilized one hour at 170° C. (dry heat). Five cubic centimeters of medium are then run in from a pipette through the side arm. The bulb is of such capacity that these 5 c.c. lie well below the inlet of the side arm. After a period of three intermittent sterilizations in the Arnold, the bulb is ready for inoculation. This is easily made by tipping until the medium runs into the cup of the side arm and there it is inoculated with a loopful of the culture to be studied. After the inoculated liquid has been allowed to drain back into the bulb the side arm is fused off in a blast lamp. If care is used to draw the seal off gradually to a point and to avoid too blunt an end, there is little danger of its cracking even if poorly annealed. The next step is the exhaustion. This is done with the Boltwood pump until, as described above, the exhaustion of the bulb is practically complete. The connecting tube is now fused at the constriction in such a way that there is left a long tapering seal. After the period of incubation the gas which has been formed is collected as follows: The tapering seal is first scratched with a diamond and then connection is made with the Antropoff pump, as previously described. The pump is next exhausted and when this is complete to below 0.01 mm. the connecting stopcock is closed and the tip of the seal on the fermentation bulb is broken by a sharp turn. The gas is now allowed to flow slowly into the pump by carefully opening the cock *f*, Fig. 3. The receiver for the gas is illustrated in Fig. 5 and needs little explanation. Before filling it with mercury the interior is moistened with water in order that the gas when collected may become saturated before analysis. To fill the receiver with mercury the air is aspirated from *G* through *H*. This outlet is buried under mercury, so that it is sealed against entrance of air both during collection of gas from the pump, and during transference to the burette through a connecting capillary tube.

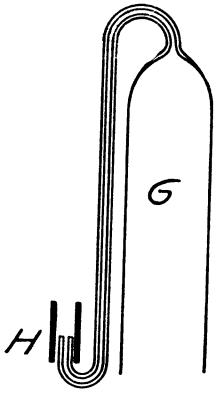


FIG. 5.—Gas receiver.

Just as in the preliminary evacuation with the Boltwood pump, so in the collection of the gas, a stopcock is used to shut off the continual distillation of water. This cock is opened for a moment only after every third or fourth stroke of the pump. The drying tube is like that previously described. It might be thought that the use of sulfuric acid as a drying agent for gas containing a large percentage of carbon dioxid would be objectionable because concentrated sulfuric acid is an even better absorbent of CO_2 than water. But there is no reason to believe that at the vacuum

attained there is any appreciable amount of carbon dioxid retained. Two burettes were used for the analysis, one of 30 c.c. and one of 15 c.c. capacity, both finely calibrated. The Hempel pipettes were of 20–30 c.c. capacity. The device for connecting a burette with a pipette is shown in Fig. 6. Altho it is essentially the same in principle as the device described in Hempel it possesses a distinct advantage for working with small volumes of gas in that there are no additional stopcocks with which the gas may come in contact. The bore of the connecting capillary is but 0.5 mm. and, fortunately, a very neat T-joint was made so that the separation of gas and absorbent at this point could be made with a high degree of accuracy.

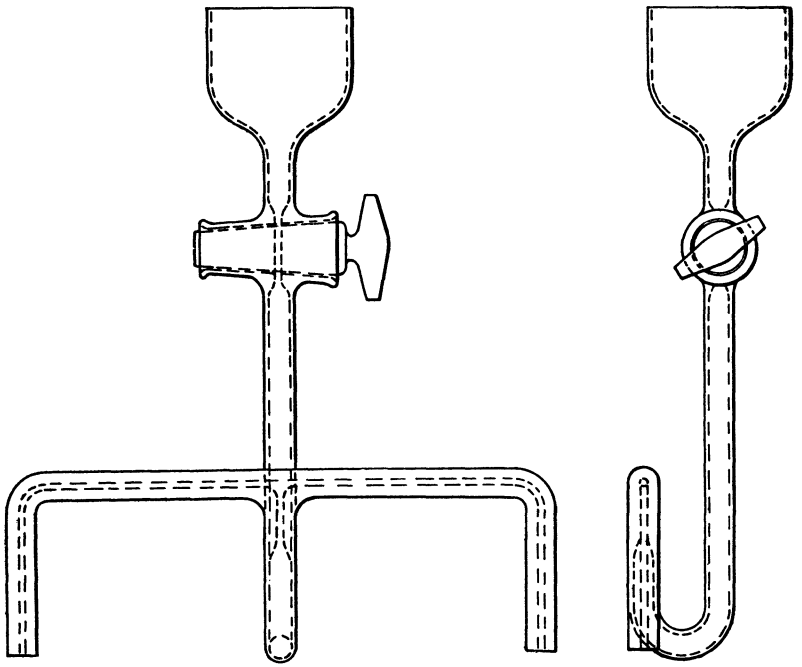


FIG. 6.—Attachment for separating gas from absorbent.

All the analyses were made over mercury. For the estimation of combustible gases, explosion with oxygen was alternated by burning in oxygen with a heated platinum spiral as recommended by Dennis and Hopkins.

Inasmuch as the volume of hydrogen estimated by the contraction after combustion came to within about 0.05 c.c. of the residual gas left after absorption of CO_2, and inasmuch as the gas after combustion showed no further contraction when placed over KOH in the many cases tried, it may reasonably be assumed that the combustible gas was in all cases exclusively hydrogen. Therefore in the majority of analyses, when the calculated hydrogen came within a few hundredths of a cubic centimeter of the gas left after absorption with KOH, it was concluded needless to carry the analysis farther.

In all data presented the gas volumes have been reduced to 760 mm. of mercury and 0° C.

THE CONSTANCY OF RESULTS.

Considering the gas analyzed as simply end products obtained by the specific method outlined, let us see whether the results are reproduced with sufficient constancy to justify their use in diagnosis.

In the first place, duplicate determinations were found to agree, as will be seen by studying Table 9. Thus *fg*, which we received from the Hygienic Laboratory of the Public Health Service and which was designated "*B. coli communis*," gave the following:

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
(4.14	2.22	1.88	53.6	45.4	1.18)
7.50	3.93	3.54	52.4	47.2	1.10
8.27	4.34	3.93	52.5	47.4	1.10
8.72	4.57	4.15	52.4	47.6	1.10

ay gave:

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
8.06	4.40	3.64	54.6	45.2	1.21
8.46	4.60	3.81	54.4	45.0	1.21

As the work progressed it was found that the determinations fell into certain groups and that there was a marked agreement in the data of different organisms. As an example, *bs*, *bn*, *cg*, and *ct* all gave about the same amount of gas and the identical ratio 2.01. When this occurred, it was considered safe to regard these organisms as identical so far as the gas-producing powers were concerned, and duplicate determinations were omitted because of the large amount of work entailed. It is only fair to point out that this failure to make duplicate determinations in favorable cases, and the repetition of our less harmonious results, all of which we have carefully included in the tables, make the inconsistencies appear greater than they probably should have been.

Altho close agreement of duplicate determinations made at the same time indicate the accuracy of the method, it may still be true that the physiological activity of an organism may vary with successive transfers.

Toward the end of the series we repeated several determinations, which were found to be in poor agreement among themselves or with the groups to which they seemed to belong. We thus

obtained determinations between which a considerable time elapsed. But inasmuch as only the apparently abnormal cases were chosen for repetition, the comparisons are, strictly speaking, not a fair test of the issue. One case demands special notice: *cw*, in November, 1911, gave:

No. of Trans.	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c.	%	%	
12	13.61	9.51	4.10	69.9	30.1	2.31

Six months later, after 6 more transfers, *cw* gave:

No. of Trans.	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c	%	%	
18	7.21	3.76	3.36	52.2	46.6	1.12
	7.02	3.69	3.29	52.6	46.9	1.12
	7.45	3.87	3.43	51.9	46.0	1.13

It was thought at first that this might be a case of a change in gas-forming power. A dried culture of the eighth transfer, made 4 months before the ratio 2.31 was obtained, was then plated out, and with this new culture the following determinations were made:

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
7.83	4.16	3.64	53.2	46.5	1.20
7.73	3.99	3.55	51.6	46.0	1.12

Thus the eighth and eighteenth transfers, 10 months apart, are identical in gas-producing power. What the cause of the singularity in the twelfth was is not known. It is not unlikely that in making the inoculation *cv* (203–204)[1] was mistaken for *cw*, since the two agree both in total volume and ratio, and since the tube of *cv* standing next to *cw* in the rack might have been picked up by mistake.

In Table 3 are the other comparisons. The numbers in the first column are the reference numbers of Table 9. The number of the transfer from which the inoculation was made is in the second column. Since one transfer was made each month, the time elapsing between the determinations can be seen at a glance.

[1] Figures are the reference numbers in Table 9.

TABLE 3.

GAS PRODUCTION BY CULTURES AT DIFFERENT TIMES.

5 c.c. of Standard Broth Containing 1 Per Cent Dextrose, Incubated 7 Days at 30° C.

Reference No.	No. of Transfer	Designation of Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio
			c.c.	c.c.	c.c.	%	%	
6.....	6	em	7.24	3.69	3.54	51.0	48.9	1.04
7.....	6	em	7.65	3.98	3.67	52.0	48.0	1.08
8.....	7	em	0.81	0.44	0.26	54.3	32.1	1.69*
9.....	11	em	9.44	4.80	4.67	50.9	49.5	1.03
83.....	9	bc	8.02	4.43	3.56	55.3	44.4	1.24
84.....	9	bc	2.09	1.10	0.96	53.1	45.9	1.15
85.....	15	bc	7.83	4.22	3.58	53.9	45.7	1.18
55.....	22	al	8.97	4.63	4.36	52.0	48.9	1.06
56.....	22	al	9.62	5.22	4.37	54.3	45.4	1.19
57.....	31	al	7.56	4.08	3.47	54.0	45.9	1.18
73.....	11	db	8.18	4.39	3.78	53.7	46.2	1.16
74.....	17	db	7.63	4.09	3.49	53.6	45.7	1.17
118.....	14	cz	14.93	9.03	5.91	60.4	39.6	1.53
119.....	17	cz	12.54	7.59	4.95	60.5	39.5	1.53
120.....	17	cz	12.28	7.60	4.68	61.8	38.2	1.62
138.....	12	cs	17.62	11.39	6.26	64.6	35.5	1.82
139.....	19	cs	16.03	10.34	5.69	64.5	35.5	1.82
141.....	12	cy	17.39	11.46	5.90	65.9	33.9	1.94
142.....	17	cy	0.05	.....	.....	.....	.....	.....
143.....	19	cy	15.31	9.67	5.59	63.2	36.5	1.73
147.....	11	ao	16.46	11.95	4.45	72.6	27.0	2.69
148.....	11	ao	16.96	9.22	4.72	54.4	27.8	1.95
149.....	20	ao	14.79	9.14	5.61	61.8	37.9	1.63
150.....	21	ao	14.18	8.78	5.34	61.9	37.7	1.64
151.....	21	ao	14.78	9.22	5.47	62.4	37.0	1.69
154.....	23	n	12.19	8.10	4.10	66.4	33.6	1.98
155.....	23	n	10.62	6.66	3.95	62.7	37.2	1.69
156.....	33	n	13.94	9.48	4.41	68.0	31.6	2.15
158.....	13	cq	17.66	11.70	5.96	66.3	33.8	1.96
159.....	20	cq	14.41	9.50	4.89	65.9	33.9	1.94
160.....	12	cr	17.68	11.51	6.16	65.1	34.8	1.87
161.....	19	cr	14.55	9.84	4.69	67.6	32.2	2.10
166.....	12	bj	no	growth	.....	.....	.....	.....
167.....	15	bj	2.56	2.21	0.31	86.3	12.1	7.13
168.....	16	bj	13.44	9.05	4.38	67.3	32.6	2.07
169.....	17	bj	12.50	8.40	4.17	67.2	33.4	2.01
173.....	3	ev	12.90	8.62	4.16	66.8	32.3	2.07
172.....	3	ev	13.11	8.84	3.93	67.4	30.0	2.25
174.....	10	ev	12.19	8.12	4.04	66.6	33.1	2.01
211.....	57	w	22.41	15.90	6.54	71.0	29.2	2.43
212.....	57	w	13.62	9.56	3.14	69.9	23.1	3.03
213.....	57	w	21.61	15.80	5.52	73.1	25.5	2.86
214.....	57	w	13.06	9.29	3.83	71.1	29.3	2.43
218.....	14	ce	1.07	.....	.....	.....	.....	.....
219.....	16	ce	2.32	1.89	0.40	81.5	17.2	4.12
220.....	17	ce	3.54	2.75	0.76	77.7	21.5	3.62
221.....	8	fb	2.28	2.21	.....	97.0	.....	.....
222.....	13	fb	4.02	4.01	.....	99.9	.....	.....
223.....	13	fb	1.39	1.39	.....	100.0	.....	.....

* Notice the small volume of gas which may account for this high ratio.

THE GASES ANALYZED ARE ONLY END PRODUCTS.

In estimating the value of the analyses made in accordance with the method we have outlined, it must be remembered that the gases recovered may not necessarily furnish exact data on the true amounts of the gases evolved by the principal reactions of the fermentation. Secondary reactions may enter in to alter the relative quantities of the constituent gases. Harden, for instance, observed that when *B. coli* is cultivated in a medium containing asparagin, the asparagin is reduced to ammonium succinate, presumably by the hydrogen, since the volume of hydrogen recovered is correspondingly diminished. In view of this it would seem highly probable that the low percentage of hydrogen which Keyes found for *B. coli*, grown on Dolt's asparagin medium, was due to oxidation of a portion of the hydrogen by the asparagin. This seems the more probable because with a medium containing no asparagin Keyes obtained a much higher percentage of hydrogen, with asparagin medium, 63.27 per cent of CO_2 and 36.05 per cent of H_2; with broth, 55.73 per cent of CO_2 and 43.56 per cent of H_2. We have obtained similar results.

TABLE 4.

INFLUENCE OF COMPOSITION OF MEDIUM ON GAS PRODUCTION OF *fg*.

5 c.c. of Medium Incubated 7 Days at 30° C.

Vacuum Apparatus.

Medium	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c.	%	%	
Standard dextrose broth	4.14	2.22	1.88	53.6	45.4	1.18
	7.50	3.93	3.54	53.4	47.2	1.10
	8.27	4.34	3.93	52.5	47.5	1.10
	8.72	4.57	4.15	52.4	47.6	1.10
Standard dextrose broth+0.1% asparagin	7.13	4.22	2.88	59.2	40.4	1.47
1% dextrose, 0.1% asparagin, 0.5% K_2HPO_4	4.89	2.99	1.86	61.1	36.1	1.61

But if we admit oxidation of the hydrogen to be a factor in lowering the amount set free from a medium containing asparagin, we must concede the possibility of oxidation of the nascent hydrogen occurring in a medium as complex as broth. There is no doubt that reductions by growing cultures of *B. coli* and other bacteria are powerful. Escherich and Pfaundler, Emmerich, Leiningen, Grafu and Loew have even observed a reduction of iron oxid

which they attributed to the nascent hydrogen liberated in a butyric fermentation. We must therefore keep it clearly in mind that the hydrogen recovered is the quantity found as an end product.

In almost all of the analyses we have made, there is to be found a small but appreciable amount of "residual gas" which we are fully justified in calling nitrogen. This finding is in accord with all the other analyses which we have been able to discover in the literature. The large percentages frequently recorded have no doubt been largely due to the fact that no attempts were made to remove the air from the medium previous to the growth of the organisms. While fully recognizing the difficulty with which last traces of nitrogen may be removed from any liquid even on boiling, we judge that the quantity of nitrogen found is in excess of the amount which may be accounted for by that source. The average amount found for the colon group is about 0.05 c.c. or about 0.7 per cent of the total gas, which is in close agreement with the analyses of Keyes. It is known that hydrogen-producing bacteria are capable of reducing nitrates with the liberation of free nitrogen. It is therefore not improbable that some, at least, of the nitrogen observed in the gas collected originated in the trace of nitrates or nitrites which existed either as original impurity of the materials (Wherry) used in the medium or which gained entrance from the laboratory atmosphere.

On the other hand we have found about this same amount of "nitrogen" in the analysis made of the gas produced by certain bacteria which furnish no hydrogen. Here there still remains the possibility of reduction of nitrates by other agents.

Some such origin of the nitrogen appears to be more plausible than incomplete removal of atmospheric nitrogen inasmuch as no detectable amount of oxygen has been found associated with it even in cases where there was no bacterial growth.

The question of whether any CO_2 is left unremoved after the preliminary evacuation and then liberated or held back by the media when the gas is collected cannot be adequately answered.

There are but few data on the retention of gases by liquids under high vacuum, and while it is true that numerous investigations are recorded on the solubilities of carbon dioxid in water, in solution of

salts, in colloidal solutions and suspensions, and in certain specific mixtures such as blood and beer, it would be unsafe to transpose the data of any of these to a medium as complex as broth, in which the bacteria are producing not only a profound chemical change, but distinct physical alterations.

It is true that we have used in the majority of cases a medium which not only contains dibasic potassium phosphate (K_2HPO_4) but which was neutralized with sodium hydrate. It is absolutely impossible to predict the exact amount of CO_2 which would have been absorbed by this medium and retained after the first evacuation. Furthermore, determinations of this quantity in one case would not necessarily hold for another. We have therefore preferred to let this error go uncorrected, having satisfied ourselves that it is far too small to affect in any noticeable degree the relative values we have used in making correlations, and too small to make very much difference in the absolute values of the ratios.

It must not be understood, however, that this small possible error has escaped our attention. We will deal with it more fully in the presentation of other results we hope soon to publish. We have considered it advisable to neglect minor sources of error until the preliminary survey is made. Then, with fuller knowledge of the large problems, we may improve methods in the direction required.

It is found that while single determinations may sometimes vary from the mode, the averages tend to place the organism definitely in a group. The significance of the grouping is more plainly seen by plotting the values of Table 9 (for the conditions under which these values were obtained see p. 442). This plot is given in Fig. 7 in which the abscissae represent volumes of carbon dioxid and ordinates volumes of hydrogen. The group about $x=4.3$ $y=3.6$ we shall call Group I; that between $x=6$ and $x=12$, Group II, and those points beyond $x=12$, Group III. In the case of those four single determinations which seemed to constitute a new group about the point $CO_2=11.5$, $H_2=6.0$ (see Fig. 7), and which happened to have been made with the same batch of medium, new values all fell within the most thickly dotted section of Group II.

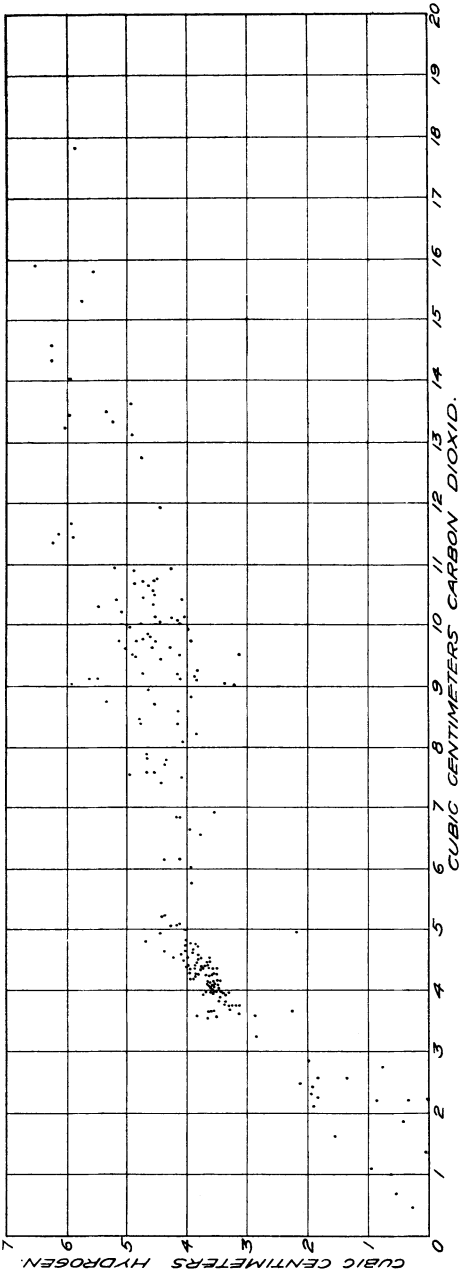


FIG. 7.—Relation of carbon dioxid to hydrogen as shown in Table 9.

Discrepancies do occur as in the comparison of *ao* and *aj*. Thus *ao* of Group II gave in the fourth determination values similar to those of *aj* of Group III, and *aj* in the sixth determination gave values easily confused with those of *ao*.

TABLE 5.

COMPARISON OF THE GAS PRODUCTION OF *ao* AND *aj*.

Organism and Reference Nos.*	Experiment Number	Total Volume	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
		c.c.	c.c.	c.c	%	%	
ao 147–151	1	14.78	9.22	5.47	62.4	37.0	1.69
	2	14.18	8.78	5.34	61.9	37.7	1.64
	3	14.79	9.14	5.61	61.8	37.9	1.63
	4	16.46	11.95	4.45	72.6	27.0	2.69
	5	16.96	9.22	4.72	54.4	27.8	1.95
aj 191–196	1	20.48	14.06	5.97	68.7	29.1	2.36
	2	19.51	13.47	5.96	69.0	30.6	2.26
	3	21.08	14.60	6.23	69.3	29.6	2.34
	4	19.38	13.25	6.04	68.4	31.2	2.19
	5	23.88	17.85	5.87	74.7	24.6	3.08
	6	14.94	9.76	5.11	65.3	34.2	1.91

*See Table 9.

Similarly, two determinations with *w* (Table 9), fall in Group II altho their ratios and the complete returns in two other cases place *w* clearly in Group III.

In the dense and well consolidated Group I, the most uniform results were obtained; but it was also the members of this group which contributed most to the area of scattered dots in the plot between I and the origin. For instance *em*, a colon obtained originally from Kral, gave in three analyses 6, 7, and 8, values which fell within the Group I, while in a fourth, Experiment 9, it gave only 0.81 c.c. of gas. A similar but less prominent case is that of *fg*, 30–33, a colon which we have studied rather extensively. The tendency among them to adhere to the $x=y$ line, together with the fact that the long axis of Group I has the slope of the $x=y$ line, would confirm the opinion that the low values are simply those of inhibited cultures which, if unobstructed, would have reached the values of Group I along the same route.

The exact nature of this inhibition is not known. It is a well known fact that cultures kept in a laboratory for a long time frequently lose something of their ability to ferment; but to ascribe this to an "attenuation" is merely renaming the phenomenon. Is

it comparable to those limitations of "diet" such as in the case of higher animals inhibit growth while allowing maintenance (Osborne and Mendel), or is it due to the natural selection of those individuals which are capable of thriving with the least energy requirements that laboratory conditions and media demand?

Undoubtedly, in certain instances negative tests result from poor inoculation. We have found rare instances where this was supposed to have happened, as in the case of *cy* (142) *bj* (166) and *by* (117). Dr. Erwin Smith suggests that the occasional failure of an organism to produce fermentation may be due to a scanty seeding; the reason being that, if a medium is unfavorable to growth, there must be a sufficient number of bacteria introduced to combat the initial toxicity. This he calls the "mass action" of bacteria.

The exact cause of the apparent "attenuations" is not known, and we have considered it best to include all these cases, since the presentation of all instances gives a fairer picture of the actual value of the data.

VARIATION DUE TO THE DIFFERENT CONDITIONS.

Attention has already been called to the variation in the ratio CO_2/H_2 for *B. coli* when grown in different media. The case mentioned was explained on the assumption that some of the hydrogen was oxidized. On the other hand, Mendel observed a marked change in ratio with increase in the concentration of sugar. With the higher concentrations less gas was obtained and with decrease in total gas, Mendel observed a decrease in percentage of carbon dioxid. In Table 6 are comparisons between Mendel's determinations and those we have made.

The change noted by Mendel is easily explained by the fact that he drew for analysis only the gas collected above the medium. Consequently when the total volume of gas was small, as both observers have found with high concentrations of sugar, a larger percentage of carbon dioxid remained dissolved than when the total volume of gas was large, and this dissolved carbon dioxid Mendel neither collected nor corrected for.

As the table will show, we have been unable to confirm the change in ratio noted by Mendel. Indeed, we have found that

when the ratio of carbon dioxid to hydrogen is in the neighborhood of 1.1–1.2 for dextrose broth, this ratio is constant under many varying conditions. On the other hand, the higher ratios are more likely to vary.

Thus while the ratio for *fg* varied but slightly with change in the concentration of the sugar, Table 7 will show considerable variation in the ratio of *aj*.

TABLE 6.

INFLUENCE OF SUGAR CONCENTRATION UPON GAS PRODUCTION BY *B. Coli Communis*.

DEXTROSE CONTENT	AFTER MENDEL				BY PUMPING (5 C.C. MEDIA USED)			
	Total Gas	CO_2	H_2	Ratio CO_2/H_2	Total Gas	CO_2	H_2	Ratio CO_2/H_2
%	c.c.	%	%		c.c.	%	%	
0.5	84.7	31.25	67.23	0.46	7.60	53.80	43.15	1.17
1.0	83.9	30.39	68.58	0.44	7.16	52.70	46.90	1.12
3.0	84.2	30.28	68.43	0.44	5.20	52.75	45.90	1.15
6.0	99.4	28.65	70.39	0.41	4.71	54.15	45.45	1.19
10.0	51.9	26.58	72.66	0.36	5.30	54.75	45.55	1.20
18.0	9.7	10.30	89.70	0.11	.....	.....	.....	.....
20.0	.....	.....	.....	.....	3.75	53.30	44.00	1.21

TABLE 7.

INELUENCE OF SUGAR CONCENTRATION UPON GAS PRODUCTION BY *aj*.

5 c.c. of Medium Incubated 7 Days at 30° C.

Concentration of Dextrose	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
%	c.c.	c.c.	c.c.	%	%	
½	10.33	6.23	4.08	60.3	39.5	1.53
	9.90	5.65	4.18	57.1	42.2	1.35
1	19.71	13.83	5.66	69.2	29.9	2.33 averages
6	16.62	12.45	4.18	74.9	25.1	2.98
10	15.53	12.00	3.51	77.3	22.6	3.42
	16.43	12.24	3.65	74.5	22.2	3.36
20	14.13	10.35	2.84	73.3	20.1	3.64
	13.10	10.24	2.88	78.2	22.0	3.56

Again, if we compare *fg*, a colon, with *ev* (bacillus of dysentery) and *aj*, we shall find little effect of incubation temperature upon *fg*, but a very noticeable effect upon *aj*.

Attention should be called to the fact that the amount of gas is doubtless an indication of the ability of the organism to grow at certain temperatures and not an influence on the function of gas formation alone. For instance, *aj* has an optimum temperature below 37° C. and grows at this temperature only sparsely.

It might be argued that the 7 days' incubation was insufficient to allow the activities of the organism or their liberated enzymes to

come to their final rest. Were this true, and were cultures to progress unequally, the determinations would have been made at different stages. It was found, however, by following the rate of gas production of *fg*, a colon, Group I, that its activity had almost ceased at the end of 48 hours. A determination made with *a*, of Group II, incubated 54 days, showed a slight increase of total gas over the amounts found at the end of 7 days, but the identical ratio. With *aj* of Group III, cultures incubated for different lengths of time up to 100 days gave more or less irregular results from which no definite conclusions may be drawn other than to say that the 7 days' incubation seemed sufficient, and that no advantage was apparent in longer incubations.

TABLE 8.

COMPARISON OF THE GAS PRODUCTION OF *fg*, *ev*, AND *aj*, AT DIFFERENT TEMPERATURES.

5 c.c. of Standard Medium Incubated for 7 Days.

Temp. °C.	Symbol of Organism	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
		c.c.	c.c.	c.c.	%	%	
18.....	fg	7.33	3.97	3.30	54.2	45.1	1.20
		8.20	4.45	3.69	54.3	45.1	1.21
30.....	fg	4.14	2.22	1.88	53.6	45.4	1.18
		7.50	3.93	3.54	52.4	47.2	1.10
		8.27	4.34	3.93	52.5	47.5	1.10
		8.72	4.57	4.15	52.4	47.6	1.10
37.5.....	fg	7.42	3.85	3.55	51.9	47.9	1.08
		8.47	4.36	3.99	51.5	47.1	1.09
30.....	ev	13.11	8.84	3.93	67.4	30.0	2.25
		12.90	8.26	4.16	66.8	32.3	2.07
		12.19	8.12	4.04	66.6	33.1	2.01
37.5.....	ev	9.41	6.16	3.25	65.4	34.6	1.89
		11.64	7.11	4.47	61.1	38.4	1.59
18.....	aj	15.14	10.11	5.03	66.8	33.2	2.01
		15.48	10.23	5.26	66.1	34.0	1.94
20.....	aj	20.85	14.45	6.26	69.3	30.0	2.31
30.....	aj	19.71	13.83	5.66	69.2	29.9	2.35 averages
37.5.....	aj	1.15	0.69	0.15	83.2	12.9	6.44
		1.13	0.95	0.13	84.3	11.5	7.36

On the other hand, the slow fermenting power of certain other bacteria such as *fm* (Table 21) has led us carefully to follow the progress of gas production of this organism, and it will be well to do the same with typical members of the groups already described.

The tendency of the colon bacillus to retain its characteristic ratio is exhibited, not only in the cases cited, but also in its action upon different sugars and allied compounds. On the other hand, *aj* has been found to give different ratios not only under different conditions but in its fermentation of different carbohydrates.

Much of the data upon this phase of the subject will be reserved until a projected series of experiments are completed; but attention is now directed to it because this same constancy among the lower ratios and the variability among the higher is seen in the results where uniform conditions were supposed to have obtained. Fig. 7 will show this at a glance: Group I is a well consolidated area; Group III is a sparsely dotted area. Consolidation of this might or might not have been more marked if more values had fallen within it. But Group II cannot be said to lack in number of determinations and yet these are scattered broadly.

A similar constancy in the colon group and variation in the data from organisms producing larger amounts of CO_2 was observed by Russell and Bassett.

We shall develop in a subsequent chapter a hypothesis explaining this discrepancy, but we cannot depend upon speculation when we come to use the data for purposes of correlation. We must see to what extent the greater or less variation indicated in Groups II and III renders the data unavailable.

In answer, it may be said at once that the values for any given organism are very much less variable than those which have been obtained by the older methods, and which have been used in differentiating species. In the second place, the inconstancy in the great majority of cases is within certain well defined limits. Altho there are what we may call transitional values between Groups I and II and between Groups II and III (see Fig. 7), yet these groups are rather clearly defined.

If the analyses are given their face value, and it is held that each of the determinations, or the average of each culture, represents a constant and definite characteristic of the organism concerned, then it will be found that by dividing them into species upon this basis alone there results a graded series of transitional species from one group to another. The logical conclusion would be a multiplication of species beyond the limits of convenience for systematic classification. But even if we admit these species to be real, we are forced to the conclusion that certain well defined characteristics are quantitatively established which throw the present series into groups, and that of these Group I, at least, is unequivo-

cally distinct. It therefore remains to be considered whether the transitional forms are truly such, or are mere suppositions imposed by the errors of the method of study. Enough data have been presented to show that some of the organisms furnish different ratios under different conditions. Until we know more thoroughly the subtle influences surrounding the cultivation of bacteria we cannot indulge in the excuse that failure to obtain *exactly* concordant results is due to variation in what may reasonably be assumed to be fundamental physiological processes. The marked increase in constancy exhibited by the abandonment of inexact for more exact methods justifies the prophecy that further improvements of method will not only reveal the physiological processes to be responsive in like degree to like influences, but that fixed characteristics will be established as a sound basis, not only for diagnosis but for the study of whatever variations or mutations do occur.

While, therefore, strict logic forbids our eliminating the "transitional" values, we feel justified in assuming that our data have indicated certain well defined groups. Using these groups as a basis, let us proceed to find whatever value they may have as a basis for the correlation of other cultural tests.

For the purposes of correlation we have assembled the results of determinations made under the following uniform conditions.

The medium was prepared as follows:

To 1,000 c.c. distilled water were added 4 gm. Liebig's beef extract and 10 gm. Witte's peptone. This was heated on a steam bath for 20 min., filtered, and the loss of water made up.

The acidity of a sample was determined by titration with tenth normal sodium hydrate using phenolphthalein as indicator. The whole was then neutralized with normal sodium hydrate (10 c.c. usually required 0.7 c.c. tenth normal sodium hydrate). After 5 minutes' heating the medium was again filtered, its acidity determined, and if 10 c.c. required more than 0.2 c.c. tenth normal sodium hydrate it was neutralized, heated, and filtered again. There were now added 1 per cent dextrose (Kahlbaum) and 0.5 per cent dibasic potassium phosphate (K_2HPO_4). After solution of these was complete, the medium was filtered and its volume at room temperature made up to 1,000 c.c. It was then tubed and sterilized 3 consecutive days in an Arnold sterilizer.

Exactly 5 c.c. of this medium were used in each determination. The period of incubation was 7 days, and the temperature 30° C. In Table 9 the numbers in the first column are given simply for convenience later in referring to a particular determination. The second column shows the laboratory designations of the organisms. In the three succeeding columns are, respectively, the total volumes, the volumes of

TABLE 9.

GAS PRODUCTION OF BACTERIA GROWN IN VACUUM BULBS.

5 c.c. of Standard Broth Containing 1 Per Cent Dextrose Incubated 7 Days at 30° C.

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
1	h	7.46	3.58	3.84	48.0	51.5	0.93	
2	h	7.32	3.55	3.65	48.5	49.9	0.97	
								0.95
3	f	7.09	3.58	3.50	50.5	49.4	1.02	1.02
4	cf	3.26	1.64	1.59	50.4	48.8	1.03	1.03
5	eq	8.23	3.85	3.72	46.8	45.1	1.04	1.04
6	em	7.24	3.69	3.54	51.0	48.9	1.04	
7	em	7.65	3.98	3.67	52.0	48.0	1.08	
8	em	(0.81	0.44	0.26	54.3	32.1	1.69)*	
9	em	9.44	4.80	4.67	50.9	49.5	1.03	
								1.05
10	bh	7.88	4.10	3.68	52.0	46.7	1.11	
11	bh	7.24	3.64	3.64	50.3	50.3	1.00	
								1.06
12	bg	7.80	4.07	3.64	52.2	46.7	1.12	
13	bg	7.24	3.64	3.60	50.2	49.7	1.01	
								1.07
14	p	8.39	4.39	4.01	53.3	47.8	1.09	
15	p	8.71	4.52	4.22	51.9	48.4	1.07	
								1.08
16	ax	7.37	3.83	3.49	52.0	47.4	1.10	
17	ax	7.74	3.95	3.75	51.0	48.4	1.05	
								1.08
18	bt	8.09	4.21	3.86	52.0	47.7	1.09	1.09
19	bi	5.02	2.58	2.38	51.4	47.4	1.08	
20	bi	6.94	3.65	3.28	52.6	47.3	1.11	
								1.10
21	ds	4.09	2.12	1.93	51.8	47.2	1.10	1.10
22	fp	1.31	0.66	0.60	50.2	45.6	1.10	1.10
23	bf	8.65	4.48	4.06	51.8	46.9	1.10	
24	bf	8.13	4.25	3.81	52.3	46.9	1.12	
								1.11
25	bm	8.44	4.40	4.00	52.1	47.4	1.10	
26	bm	8.31	4.32	3.97	52.0	47.8	1.09	
								1.10
27	ek	7.11	3.77	3.39	53.0	47.7	1.11	1.11
28	aq	9.40	4.94	4.41	52.6	46.9	1.12	1.12
29	aq	(3.54	.....	.....	.....	.....	.....)*	
30	fg	4.14	2.22	1.88	53.6	45.4	1.18	
31	fg	7.50	3.93	3.54	52.4	47.2	1.10	
32	fg	8.27	4.34	3.93	52.5	47.5	1.10	
33	fg	8.72	4.57	4.15	52.4	47.6	1.10	1.12
34	az	7.90	4.22	3.62	53.4	45.8	1.17	
35	az	8.19	4.19	3.96	51.2	48.4	1.08	
								1.12
36	bb	8.04	4.26	3.85	52.7	47.6	1.11	
37	bb	6.11	3.22	2.86	52.7	46.8	1.13	
								1.12
38	bp	7.17	3.74	3.30	52.2	46.0	1.13	1.13
39	dl	7.57	4.00	3.51	52.8	46.4	1.14	
40	dl	7.52	3.97	3.53	52.8	46.9	1.12	
								1.13
41	fk	8.72	4.60	4.11	52.8	47.1	1.12	
42	fk	8.56	4.54	4.00	53.0	46.7	1.14	
								1.13
43	ba	8.22	4.34	3.86	52.8	47.0	1.12	
44	ba	8.31	4.44	3.82	53.4	46.0	1.16	
								1.14
45	be	7.67	4.11	3.53	53.6	46.0	1.16	
46	be	7.74	4.05	3.60	52.3	46.5	1.12	
								1.14
47	cw	(13.61	9.51	4.11	69.9	30.1	2.32)*†	
48	cw	7.45	3.87	3.43	51.9	46.0	1.13	
49	cw	7.02	3.69	3.29	52.6	46.9	1.12	
50	cw	7.21	3.76	3.36	52.2	46.6	1.12	
51	cw	7.73	3.99	3.55	51.6	46.0	1.12	
52	cw	7.83	4.16	3.64	53.2	46.5	1.20	
								1.14

* Not averaged.

† See p. 431.

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
53	ew	9.67	5.21	4.39	53.9	45.4	1.19	
54	ew	8.29	4.27	3.95	51.5	47.6	1.08	
								1.14
55	al	8.97	4.63	4.36	52.0	48.9	1.06	
56	al	9.62	5.22	4.37	54.3	45.4	1.19	
57	al	7.56	4.08	3.47	54.0	45.9	1.18	
								1.14
58	dh	6.78	3.60	3.14	53.1	46.3	1.15	
59	dh	7.69	4.08	3.55	53.1	46.2	1.15	
								1.15
60	dr	4.63	2.48	2.15	53.6	46.4	1.15	1.15
61	du	7.35	3.83	3.34	52.1	45.4	1.15	1.15
62	ea	7.97	4.25	3.68	53.3	46.2	1.15	1.15
63	ec	8.20	4.36	3.78	53.2	46.1	1.15	1.15
64	bd	8.13	4.33	3.79	53.3	46.6	1.14	
65	bd	7.45	3.99	3.41	53.6	45.8	1.17	
66	bd	6.99	3.76	3.19	53.8	45.6	1.18	
								1.16
67	fo	8.78	4.66	4.04	53.1	46.0	1.15	
68	fo	8.96	4.81	4.10	53.7	45.8	1.17	
								1.16
69	fj	8.84	4.74	4.04	53.6	45.7	1.17	1.17
70	fj	(4.51	2.43	1.93	53.9	42.8	1.26)*	
71	aw	7.71	4.15	3.54	53.8	45.9	1.17	1.17
72	aw	7.29	3.92	3.33	53.6	45.7	1.17	
73	db	8.18	4.39	3.78	53.7	46.2	1.16	
74	db	7.63	4.09	3.49	53.6	45.7	1.17	
								1.17
75	di	8.35	4.51	3.78	54.0	45.3	1.19	
76	di	7.54	4.05	3.54	53.7	46.9	1.14	
								1.17
77	dk	8.29	4.52	3.80	54.0	45.3	1.19	
78	dk	8.27	4.41	3.87	53.3	46.8	1.14	
								1.17
79	df	7.26	3.97	3.30	54.7	45.5	1.20	
80	df	7.56	4.03	3.47	53.3	45.9	1.16	
								1.18
81	ee	8.16	4.39	3.71	53.8	45.5	1.18	1.18
82	er	8.54	4.61	3.90	54.0	45.7	1.18	1.18
83	bc	8.02	4.43	3.56	55.3	44.4	1.24	
84	bc	2.09	1.10	0.96	53.1	45.9	1.15	
85	bc	7.83	4.22	3.58	53.9	45.7	1.18	
								1.19
86	cx	9.35	5.07	4.26	54.2	45.6	1.19	1.19
87	dv	(7.68	4.10	3.58?	53.4	46.6	1.15)*	
88	dv	7.66	4.17	3.44	54.4	44.9	1.21	
89	dv	7.44	3.98	3.41	53.5	45.8	1.17	
								1.19
90	ak	8.67	4.67	3.90	53.9	45.0	1.20	1.20
91	dc	6.87	3.74	3.12	54.4	45.4	1.20	1.20
92	dg	8.04	4.39	3.63	54.6	45.1	1.21	
93	dg	7.66	4.15	3.48	54.2	45.5	1.19	
								1.20
94	ef	7.82	4.26	3.55	54.5	45.4	1.20	1.20
95	ay	8.06	4.40	3.64	54.6	45.2	1.21	
96	ay	8.46	4.60	3.81	54.4	45.0	1.21	
								1.21
97	do	7.95	4.33	3.58	54.5	45.0	1.21	1.21
98	da	8.71	4.79	3.94	55.0	45.2	1.22	1.22
99	dd	7.80	4.26	3.50	54.6	44.9	1.22	1.22
100	ej	8.09	4.45	3.61	55.0	44.6	1.23	1.23
101	es	8.61	4.75	3.86	55.2	44.8	1.23	1.23
102	eu	8.16	4.46	3.63	54.7	44.5	1.23	1.23
103	bz	9.60	4.71	3.81	49.1	39.7	1.24	1.24
104	dq	6.49	3.58	2.88	55.2	44.4	1.24	1.24
105	ed	9.20	5.10	4.13	55.4	44.9	1.23	
106	ed	9.11	5.00	4.01	54.9	44.0	1.25	
								1.24
107	eh	8.22	4.54	3.62	55.2	44.0	1.25	1.25

* Not averaged.

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
108	u	9.74	5.78	3.93	59.3	40.3	1.47	
109	u	9.35	5.09	4.18	54.4	44.7	1.22	1.35
110	el	(1.62	1.00	0.61	61.7	37.6	1.64)*	
111	el	4.45	2.57	1.88	57.8	42.2	1.36	
112	el	4.44	2.55	1.89	57.4	42.3	1.35	1.36
113	fn	(1.25	0.69	0.53	55.2	42.4	1.30)*	
114	fn	10.57	6.18	4.37	58.5	41.3	1.41	
115	fn	10.49	6.11	4.38	58.0	42.0	1.33	1.37
116	bv	11.13	6.05	3.94	54.4	35.4	1.54	1.54
117	bv	No growth						
118	cz	14.93	9.03	6.91	60.4	39.6	1.53	
119	cz	12.54	7.59	4.95	60.5	39.5	1.53	
120	cz	12.28	7.60	4.68	61.8	38.2	1.62	1.56
121	ck	12.14	7.60	4.53	62.6	37.3	1.68	1.68
122	cl	12.55	7.84	4.68	62.5	37.3	1.68	1.68
123	m	10.41	6.59	3.77	63.3	36.2	1.75	
124	m	11.03	6.87	4.14	62.3	37.5	1.66	1.71
125	s	12.14	7.73	4.37	63.7	36.0	1.77	
126	s	10.97	6.87	4.13	62.6	37.6	1.66	1.72
127	dp	13.23	8.49	4.79	64.2	36.2	1.77	
128	dp	12.45	7.91	4.69	63.5	37.7	1.69	1.73
129	o	10.38	6.18	4.13	59.5	39.8	1.50	
130	o	10.45	6.94	3.51	66.4	33.6	1.98	1.74
131	fl	11.91	7.42	4.42	62.3	37.1	1.68	
132	fl	12.12	7.83	4.35	64.1	35.6	1.80	1.74
133	cj	13.20	8.41	4.79	63.7	36.3	1.76	1.76
134	cj	(8.24	.....	.....	.....	.....	.....)*	
135	c	(7.19	4.95	2.20	68.8	30.6	2.25)*	
136	c	12.26	7.84	4.36	64.0	35.6	1.80	1.80
137	c	4.86	2.86	2.00	58.8	41.2	1.43)*	
138	cs	17.62	11.39	6.26	64.6	35.5	1.82	
139	cs	16.03	10.34	5.69	64.5	35.5	1.82	1.82
140	cu	15.01	9.66	5.28	64.4	35.2	1.83	1.83
141	cy	17.39	11.46	5.90	65.9	33.9	1.94	
142	cy	(0.05	.....	.....	.....	.....	.....)*	
143	cy	15.31	9.67	5.59	63.2	36.5	1.73	1.84
144	b	11.75	7.50	4.10	63.9	34.9	1.83	
145	b	13.40	8.73	4.63	65.1	34.6	1.88	1.86
146	en	15.85	10.33	5.49	65.2	34.6	1.88	1.88
147	ao	14.78	9.22	5.47	62.4	37.0	1.69	
148	ao	14.18	8.78	5.34	61.9	37.7	1.64	
149	ao	14.79	9.14	5.61	61.8	37.9	1.64	
150	ao	16.46	11.95	4.45	72.6	27.0	2.69	
151	ao	16.96	9.22	4.72	54.4	27.8	1.95	1.92
151	de	14.69	9.64	5.01	65.6	34.1	1.92	1.92
153	bw	13.66	8.96	4.64	65.6	34.0	1.93	1.93
154	n	12.19	8.10	4.10	66.4	33.6	1.98	
155	n	10.62	6.66	3.95	62.7	37.2	1.69	
156	n	13.94	9.48	4.41	68.0	31.6	2.15	1.94
157	bq	14.40	9.53	4.90	66.2	34.0	1.94	1.94
158	cq	17.66	11.70	5.96	66.3	33.8	1.96	
159	cq	14.41	9.50	4.89	65.9	33.9	1.94	1.95

* Not averaged.

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
160	cr	17.68	11.51	6.16	65.1	34.8	1.87	
161	cr	14.55	9.84	4.69	67.6	32.2	2.10	
								1.99
162	bs	15.63	10.41	5.17	66.6	33.1	2.01	2.01
163	bu	14.60	9.76	4.85	66.9	33.2	2.01	2.01
164	cg	15.31	10.21	5.08	66.7	33.2	2.01	2.01
165	ct	14.94	9.99	4.96	66.9	33.2	2.01	2.01
166	bj	No growth						
167	bj	(2.56	2.21	0.31	86.3	12.1	7.13)*	
168	bj	13.44	9.05	4.38	67.3	32.6	2.07	2.04
169	bj	12.50	8.40	4.17	67.2	33.1	2.01	2.06
								2.11
170	dm	14.56	9.79	4.75	67.2	32.6	2.06	
171	by	14.95	10.05	4.77	67.2	31.9	2.11	
172	v	13.11	8.84	3.93	67.4	30.0	2.25	
173	v	12.90	8.62	4.16	66.8	32.3	2.07	2.11
174	v	12.19	8.12	4.04	66.6	33.1	2.01	
175	bl	14.43	9.81	4.61	68.0	31.9	2.13	2.13
176	br	14.26	9.73	4.52	68.2	31.7	2.15	2.15
177	cc	16.12	10.96	5.02	68.0	31.1	2.18	2.18
178	fd	15.55	10.70	4.89	68.8	31.5	2.19	2.19
179	bk	13.44	9.21	4.17	68.6	31.0	2.21	2.21
180	fc	15.20	10.47	4.73	68.9	31.1	2.21	2.21
181	dw	13.40	9.16	4.11	68.4	30.7	2.23	2.23
182	fe	15.78	10.93	4.89	69.3	31.0	2.24	2.24
183	cn	15.22	10.94	4.28	71.9	28.6	2.25	2.25
184	ey	14.69	10.15	4.51	69.1	30.7	2.25	2.25
185	cb	14.90	10.08	4.45	67.6	29.9	2.26	2.26
186	cm	15.51	10.73	4.74	69.2	30.6	2.27	2.27
187	ch	15.09	10.51	4.55	69.7	30.1	2.31	2.31
188	dz	15.22	10.59	4.57	69.6	30.0	2.32	2.32
189	dj	15.30	10.67	4.64	69.7	30.3	2.30	
190	dj	15.30	10.75	4.55	70.3	29.7	2.36	
								2.33
191	aj	20.48	14.06	5.97	68.7	29.1	2.36	
192	aj	19.51	13.47	5.96	69.0	30.1	2.26	
193	aj	21.08	14.60	6.23	69.3	29.6	2.34	
194	aj	19.38	13.25	6.04	68.4	31.2	2.19	
195	aj	23.88	17.85	5.87	74.7	24.6	3.04	
196	aj	14.94	9.76	5.11	65.3	34.2	1.91	
								2.35
197	a	14.55	10.42	4.10	71.6	28.2	2.54	
198	a	19.02	13.53	5.32	71.1	28.0	2.54	
199	a	13.15	9.19	3.88	69.9	29.5	2.37	
200	a	12.06	8.24	3.85	68.3	31.9	2.14	
								2.40
201	et	15.30	10.79	4.50	70.5	29.4	2.40	2.40
202	bo	14.28	10.09	4.15	70.7	29.1	2.43	2.43
203	cv	12.91	9.11	3.83	70.6	29.7	2.38	
204	cv	13.68	9.76	3.94	71.3	28.8	2.48	
								2.43
205	dn	14.49	10.15	4.28	70.0	29.5	2.37	
206	dn	14.01	9.97	4.00	71.2	28.5	2.49	
								2.43
207	ca	14.18	10.06	4.13	70.9	29.1	2.44	2.44
208	ap	18.92	13.36	5.25	70.6	27.7	2.54	
	ap	18.03	12.76	4.77	70.8	26.5	2.67	
								2.61
209	ag	(16.21	.....	.....	.....	.....	.....)*	
210	ag	17.94	13.12	4.93	73.1	27.5	2.66	2.66
211	w	22.41	15.90	6.54	71.0	29.2	2.43	
212	w	13.62	9.52	3.14	69.9	23.1	3.03	
213	w	21.61	15.80	5.52	73.1	25.5	2.86	
214	w	13.06	9.29	3.83	71.1	29.3	2.43	
								2.69
215	ff	18.66	13.64	4.91	73.1	26.3	2.78	
216	ff	21.35	15.34	5.79	71.9	27.1	2.65	
								2.72

TABLE 9—*Continued.*

Reference No.	Culture	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2	Average Ratio
		c.c.	c.c.	c.c.	%	%		
217.....	ci	12.25	9.02	3.20	73.7	26.1	2.82	2.82
218.....	ce	(1.07	.....	.....	.....	.....	.....)*	
219.....	ce	2.32	1.89	0.04	81.5	17.2	4.12	
220.....	ce	3.54	2.75	0.76	77.7	21.5	3.62	
221.....	fb	2.28	2.21	0.00	97.0	00.0		3.87
222.....	fb	4.02	4.01	0.00	99.9	00.0		
223.....	fb	1.39	1.39	0.00	100.0	00.0		

* Not averaged. † See p. 431.

carbon dioxid, and the volumes of hydrogen all reduced to 0° C. and 760 mm. The percentages of carbon dioxid, and of hydrogen in the next two columns are followed by the values of the ratios CO_2/H_2.

The determinations have been arranged in Table 9 in the order of the ascending average values of the ratio.

RELATIONSHIP BETWEEN THE GAS-PRODUCING POWERS OF THE DIFFERENT GROUPS.

It has been noticed that with the ascent of the ratios there is a very marked increase in the total volumes of gas, and that this is due almost entirely to an increase in carbon dioxid. In other words, the volume of hydrogen is more or less constant. This is shown strikingly in the plotted values, Fig. 7. In this figure the abscissae represent volumes of carbon dioxid and the ordinates volumes of hydrogen. Each point in the plot represents the carbon dioxid and hydrogen of one of the determinations listed in Table 9. Omitting a consideration of the area of scattered dots nearest the origin of the plot which we have previously explained, it will be seen that there is a very marked tendency toward constancy of hydrogen with but very slight increase in the volume of hydrogen to correspond to a marked increase in carbon dioxid. A plot made with only average values emphasizes this to some extent.

A similar relation between the total volume of gas and the value of carbon dioxid is to be found in the work of MacConkey. A recast of his Table 1, according to ascending quantities of gas is given:

Ratio CO_2/H_2.	1/5	1/4	1/3	1/2	2/3	3/2	2/1
Volume of gas modes at.....	25%	30%	25% and 40–50%	40% 50 " 60 "	60%	87%	90%

This relation, altho not so distinct, is still evident in MacConkey's Table 2 in which are compared a variety of bacteria from various sources. Smith noted a similar relationship between the gas production of *B. coli* and *B. cloacae* and the same may be discovered in the comparisons of Burri and Düggeli. One must be very careful, however, not to confuse this with the *apparent* increase of carbon dioxid over hydrogen which takes place after the medium in a fermentation tube has become saturated with gas and the carbon dioxid is no longer held back.

The fact that even with accurate methods Groups II and III continue to show slight variation in the relation CO_2/H_2, whatever the cause may be, is sufficient evidence that part, at least, of the carbon dioxid is liberated independently of the hydrogen. Were it not so, some constant relationship between the volumes of each gas would be apparent. That such a relationship does exist in the case of the colon group and that this relationship is indicated by the ratio $CO_2/H_2=\frac{1}{1}$ can be made to appear reasonable. Hoppe-Seyler's observation in 1887 that calcium formate infected with river mud is decomposed into carbon dioxid and hydrogen; Scruel's contention that the gases evolved by *B. coli* are the products of the reaction $HCOOH \rightarrow H_2+CO_2$; and the more thorough investigations of P. F. Frankland and his collaborators, of Pakes and Jollyman, of Harden, and, recently, of Franzen and his collaborators have made it quite probable, that not only *B. coli*, but a large number of bacteria are capable of decomposing the formic acid which they produce into equal volumes of carbon dioxid and hydrogen. Were this the only reaction involved, it would lead to the conclusion that the ratio CO_2/H_2 should be 1/1 during the whole course of the fermentation. Various observers have maintained that the ratio for the colon bacillus is not constant. Stamm, for instance, argues that the CO_2 and H_2 are evolved separately, for he observed that at the beginning of the fermentation there was more carbon dioxid in the gas collected above the medium than later in the fermentation. The gas remaining in solution, if added to this, would have served only to accentuate this difference. But attention has already been called to the possibility of loss of hydrogen by oxidation. On the other hand, Mendel observed a marked change of

ratio with varying concentrations of sugar. This conclusion would somewhat invalidate the theory that the gases liberated by *B. coli* are the products of the reaction $H_2CO_2 \rightarrow H_2 + CO_2$.

As indicated in a previous paragraph, we have not only shown Mendel's results to be of no value, but have found the ratio to be practically unchanged by addition of sugar. It was also shown to be unchanged by variations in the temperature of incubation.

This constancy which we have observed in the ratio for *B. coli* is at least suggestive of a single chief reaction and a close relationship between the carbon dioxid and hydrogen. It would indeed be rash to push this hypothesis beyond the point where it is useful as a temporary foundation upon which to build the plans of future research. But considering it as such, we may go a step farther. The easy gradations illustrated in the plot, Fig. 7, with which Groups II and III lead off from Group I, and the remarkable coincidence that the hydrogen volumes of all the groups are approximately the same make plausible the assumption that the reaction $HCOOH \rightarrow H_2 + CO_2$ holds good for all, and that superimposed upon this in the fermentation by Groups II and III are other reactions in which is evolved only carbon dioxid.

The justification for this hypothesis may be divided into two parts. It already has been made the guide in a search for those substances which the members of Groups II and III decompose with the liberation of CO_2 alone, in the hope that the use of these substances may prove to be a rational means of differentiation. In the second place, the hypothesis serves in taking the first step toward an explanation of certain variations in gas production which may be attributed to variations in the conditions of growth rather than to instability in the phsyiological powers of the bacteria.

Assuming that the gases produced are the product of a single reaction, the ratio of these gases must remain constant whatever the condition. Such is the tendency exhibited by members of Group I. But if two or more reactions are involved, and these are in any way independent, it is perfectly possible that one or all will be subject to variation to different degrees as the conditions surrounding them vary. In this case, the conditions must be rigidly constant to obtain the same ratio of gases in any two cases. The

conduct of *aj*, and the members of Groups II and III point toward the validity of this explanation of the variations. If we accept this hypothesis we see at once that very slight changes in the conditions might affect the extent to which the one reaction would outrun the other. The surprising point would then be not that the ratios varied but that they were so constant.

We wish in particular to emphasize the remarkable tendency in our series for the hydrogen volume to remain constant. This throws upon the CO_2 the responsibility for the change in ratio. Indeed we could almost as well group the organisms upon the basis of their CO_2 production alone, and *it is the CO_2 which the Smith fermentation tube determined with least accuracy.*

CORRELATION OF RESULTS.

In attempting to arrange a collection of bacteria in any semblance of order the question of methods of correlating the mass of heterogeneous observations is of the greatest importance.

So far as systematic bacteriology is concerned the most complete set of records of the characters of various cultures merely adds to the confusion if we are unable so to arrange and classify them that they aid us in grouping the cultures according to their natural relationships. At the very beginning we are confronted with the problem of how distinctly the bacteria are segregated by nature into any grouping that may be considered as genera, species, and varieties. The earlier systematic bacteriologists, following the botanists, with whom they were closely affiliated, divided the bacteria on an almost entirely morphological basis, a plan which the later bacteriologists have for the most part attempted to follow while recognizing the impracticability of carrying it to its logical conclusion. In the earlier days the description of species and construction of genera were based largely on descriptions of isolated cultures with little regard for the real relationship to each other. More recently, however, there has been a tendency to recognize certain more or less well defined groups possessing many fundamental characters in common and doubtless descended from common ancestors.

These groups probably have no sharp limits. Even the higher and more complex plant and animal species are not separated from one another by a sharp line but merge imperceptibly with their nearest relatives on each side. The bacteria, with their simple structure and rapid reproduction, are much more prone to variation, and it is probable that while defined natural groups exist they are surrounded by atypical forms connecting them with other equally distinct groups. How shall we study our cultures to determine the characters, limits, and subdivisions of these major groups? On which characteristics shall we depend for the separation of one from another? To the latter question no categorical answer can be given since the system must be fitted to the group rather than the group to the system. Characters which may be of fundamental importance in classifying the bacteria of one group may be of no value in another. In any attempt at classification each group must be studied, if the information is not already available, to determine which characters are significant and which are valueless.

Of perhaps even greater importance is the manner in which we use our information to form our groups. We may use it to create a confusion from which there appears no hope of forming groups, natural or artificial; we may form arbitrary groups or we may so arrange it that the groups, genera, species, or whatever we may choose to call them, follow the lines of natural descent and relationship. Obviously the larger groups will have a considerable number of characters in common while the subgroups will be connected by the common possession of characters the number and character of which is still a matter of opinion. The usual procedure is to make the final separation on the basis of the presence or absence of a single character. This is illustrated by the method of classification proposed by the standard card of the American Bacteriologists in which two cultures may be widely separated by a difference in a single character. Thus a culture which through degeneration has lost the power of liquefying gelatin is entirely removed from a culture with which it is identical except that the liquefaction of gelatin is retained and the two cultures, tho closely related, are

thrown into separate groups. While the card is useful as a means of recording the characters of bacterial cultures it can never be successful as a method of classification, because it assumes that the significant characters are identical for all groups of bacteria. Furthermore, the practice of making divisions on a single character, sometimes of questionable stability, can only bring systematic bacteriology to such confusion that it is not surprising that some bacteriologists doubt the existence of real species among the bacteria. The possibilities of any system of classification in which final separations are made on the basis of single characters is well illustrated by the arrangement of the colon-aerogenes group, by Bergey and Deehan, who by using 8 characters made 256 possible varieties of which they found 43.

Another good illustration of the arbitrary method of classification is found in the revision of the colon group by Jackson, adopted by the American Public Health Association. In this classification the group is divided into two subgroups, one fermenting dulcite and containing *B. coli communior* and *B. coli communis*, and one failing to ferment dulcite, including *B. aerogenes* and *B. acidi lactici*. *B. communior* is separated from *B. communis* by the ability to ferment saccharose and *B. aerogenes* from *B. acidi lactici* in the same way. Each of these species is farther divided into a large number of varieties on the basis of mannite and raffinose fermentation, indol production, nitrate reduction, and other minor characters. One variety liquefies gelatin, altho it is specified in the list of common characters distinguishing the group that gelatin is not liquefied.

It may be very convenient to have a classification in which a culture can be definitely placed by means of a few tests but it is doubtful if this can be considered more than an expedient if it is not based on the sound foundation of natural relationship. If these separations are not, as they seem to be, on a purely arbitrary basis but follow natural lines, we should expect that if we arrange our cultures by this system each of the four species would be further distinguished by the distribution of the characters which we have found to be constant and distinctive.

Using as distinguishing characters the gas ratio, the fermentation of adonite, starch, and glycerin, the production of indol, and the reduction of nitrates, we obtain Table 10.

TABLE 10.

CORRELATION IN SPECIES OF THE COLON GROUP.

SPECIES	SIGNIFICANT CHARACTER		NUMBER OF CULTURES	CO_2-H_2 RATIO		ADONITE		STARCH		GLYCERIN		INDOL		NITRATES	
	Dulcite	Saccharose		Above 1.4	Below 1.4	+	−	+	−	+	−	+	−	+	−
Communior	+	+	26	13	13	11	14	12	14	6	20	24	2	25	1
Percentage of total				50.0	50.0	44.0	56.0	46.15	53.85	23.08	76.92	92.31	7.69	96.15	3.85
Communis	+	−	16	0	16	0	16	0	16	10	6	13	3	15	1
Percentage of total					100		100		100	62.50	37.50	81.25	18.75	93.75	6.25
Aerogenes	−	+	40	31	8	24	16	19	21	11	29	27	13	39	1
Percentage of total				79.49	20.51	60.0	40.0	47.50	52.50	27.50	72.50	67.50	32.50	97.50	2.50
Acidi lactici	−	−	25	5	19	6	19	0	25	10	15	19	5	22	3
Percentage of total				20.83	79.17	24.0	76.0		100	40.0	60.0	79.17	20.83	88.0	12.0

Of the 26 cultures of our collection that could be classed as *B. communior* we find that one-half have a gas ratio below 1.4, about one-half ferment adonite and starch, 76 per cent fail to ferment glycerin, and nearly all form indol and reduce nitrates. On the other hand, of the 16 cultures which could be designated as *B. communis* all have a ratio below 1.4, all fail to ferment adonite and starch, while 62 per cent ferment glycerin, and nearly all form indol and reduce nitrates.

Forty cultures were classed as *B. aerogenes*, of which 79 per cent had a gas ratio above 1.4. Nearly all of these reduced nitrates but there was little or no evidence of correlation in any of the other tests. A large part of the 25 cultures classed according to this scheme as *B. acidi lactici* have a gas ratio below 1.4; 76 per cent fail to ferment adonite, none ferment starch, and 40 per cent ferment glycerin. If these reactions indicate relationship *B. acidi lactici* should be grouped with *B. communis* rather than with *B. aerogenes*. On the whole these comparisons tend to show that the scheme of classification adopted by the American Public Health Association is based on arbitrary distinctions and, with the exception of *B. communis*, which by an accident seems to be a natural

group, the species created may still contain a heterogeneous collection of organisms.

THE BIOMETRICAL METHOD.

The close relationship shown by the few cultures classified as *B. communis* gives a good illustration of the method which may be used to establish the groups into which the larger group has been divided by nature. A natural group or species is distinguished not by a single peculiar character but by several which have been developed simultaneously under the stress of changing conditions. The type of this group occurs with great frequency while the variants, those who have lagged behind or forged ahead in the development, are found with less and less frequency as they are more and more removed from the type. The study of a large number of cultures with the proper tabulation of the results to show the frequency of occurrence of various combinations of characters as they indicate relationship is the essence of the biometrical method as applied to bacteriology. No particular set of culture characters is required. On the contrary, it is essential to the success of the method that the characters be selected with special reference to the general nature of the bacteria studied. Those that are useful in one group may be of no value in another. This method has been used, unconsciously, by bacteriologists for many years in the gradual establishment of groups with uncertain boundaries of which the colon group is a good example. It is only when it appears under a name of its own that it is looked upon as an innovation. The objection, which may possibly be made, that the species which this method would create would not be sharply defined is a criticism, not of the method by which the species are described, but of the condition in which they exist. A method which will enable us to describe actual species must, necessarily, leave the limits somewhat uncertain, since in nature no species, and especially no bacteriological species, can have hard-and-fast limits.

A botanist selects without much difficulty a type plant from which he makes a description of the species. He usually has before him a large number of plants from which he can choose, not one stunted by unfavorable soil or abnormally large from the influence

of exceptional circumstances, but one which is evidently a fair representative of all those before him. The bacteriologist, on the other hand, sees only the culture with which he is working. To determine the typical culture he must work over a large number of cultures and arrange them in the order of their frequency. The accumulated results of many years' work has established the type and, in an indefinite way, the limits of the colon-aerogenes group.

It is proposed in this paper to point out the existence within the larger group of well defined subgroups marked by co-ordinated characters and separated from the other sub-groups by distinctive characters. Howe, who studied 630 cultures from the stools of 20 individuals, found that this collection was about equally divided between 2 groups differing principally in the fermentation of saccharose and raffinose. The amount of gas and gas ratio was not used, as he considered this of no value. He also states that there is no correlation between mannite, dulcite, starch and other tests and that indol, ammonia, and the nitrates are of no vlaue.[1]

While Howe's two groups no doubt represent the division which could be made in the colon group on the basis of these two tests, there is nothing in the brief abstract available to show that numerous subgroups do not exist within the two groups formed.

It has already been shown that when accurately determined the CO_2/H_2 ratio brings the cultures together in a few circumscribed areas.

By arranging the cultures to show frequency of occurrence, we obtain Table 11 and Fig. 8.

TABLE 11.

DISTRIBUTION OF CULTURES IN RELATION TO CO_2:H_2 RATIO.

0–1	1–1.1	1.1–1.2	1.2–1.3	1.3–1.4	1.4–1.5	1.5–1.6	1.6–1.7	1.7–1.8	1.8–1.9	1.9–2.0	2.0–2.1	2.1–2.2	2.2–2.3	2.3–2.4	2.4–2.6	2.6–2.7	2.7–2.8	2.8–2.9	2.9–3.0	3.0 Up
1	12	37	11	3	0	2	2	7	5	7	6	6	9	4	5	0	3	1	1	1

This shows that a large number of cultures have in common a gas ratio varying within narrow limits and sharply separated from the gas ratio of the other cultures of this collection. In other

[1] In the abstract published in *Science*, the first subgroup of Group II is identical with the second subgroup of Group I. This is evidently a typographical error.

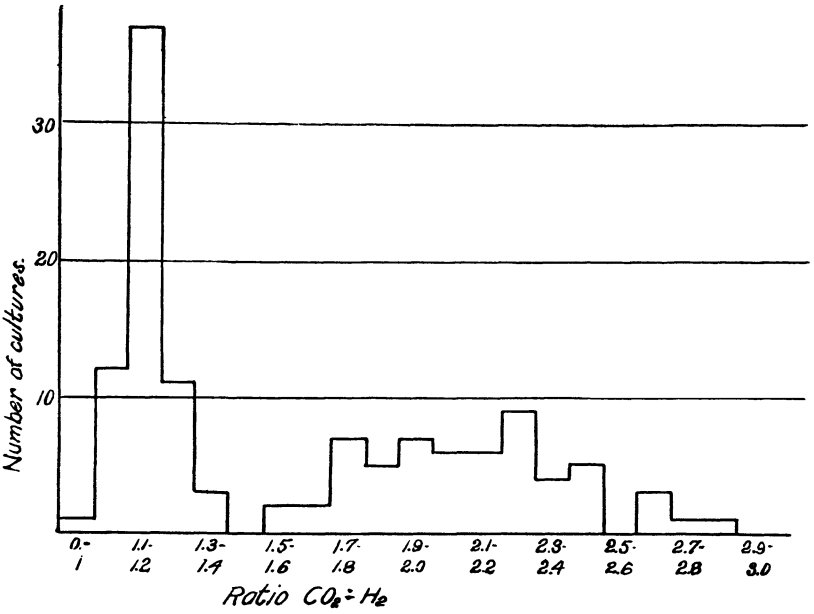


FIG. 8.—Frequency curve for $CO_2 : H_2$ ratio.

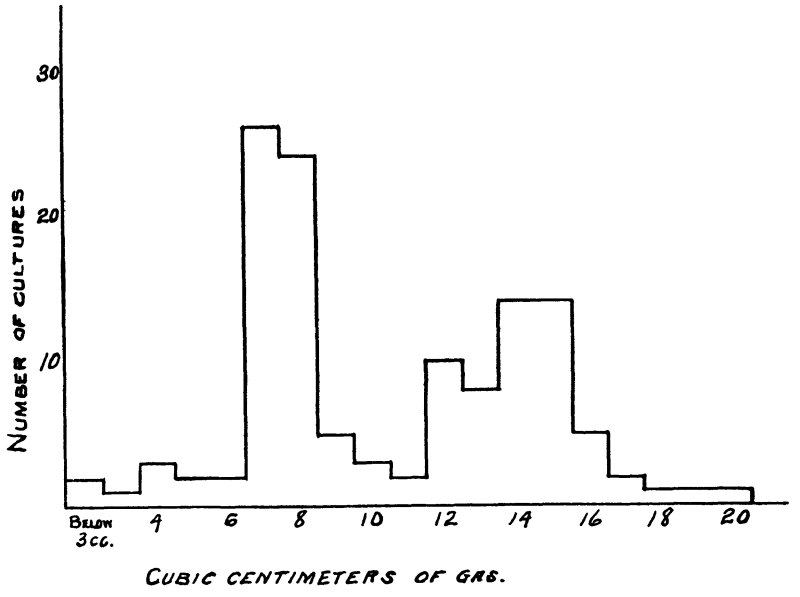


FIG. 9.—Frequency curve for amount of gas.

words, a large number of these cultures bring about an identical or at least very similar reaction when dextrose is fermented. In a similar way we obtain Table 12 and Fig. 9 by arranging the number of cultures forming certain amounts of gas under definite conditions.

TABLE 12.

DISTRIBUTION OF CULTURES IN RELATION TO AMOUNT OF GAS FORMED.

Below 3 c.c.	3 c.c.	4 c.c.	5 c.c.	6 c.c.	7 c.c.	8 c.c.	9 c.c.	10 c.c.	11 c.c.	12 c.c.	13 c.c.	14 c.c.	15 c.c.	16 c.c.	17 c.c.	18 c.c.	19 c.c.	20 c.c.
2	1	3	2	2	26	24	5	3	2	10	8	14	14	5	2	1	1	1

We find here also a sharp mode at 7–8 c.c. of gas and one, perhaps two, between 12 and 18 c.c. By going through the original tables we find, as is shown in Table 13, that of the 65 cultures

TABLE 13.

RELATION OF CO_2 : H. RATIO TO AMOUNT OF GAS.

RATIO	NUMBER OF CULTURES	CUBIC CENTIMETERS OF GAS			
		Up to 9.9	10–13.9	14–15.9	16 Up
0.95–1.37.....	65	65 100%	0	0	0
1.5–2.0.....	24	0	15 62.5%	9 37.5%	0
Above 2.....	35	0	6 17.2%	23 65.6%	6 17.2%

which form the mode over 1.1–1.2 in the gas ratio curve, 100 per cent form less than 10 c.c. of gas under the predetermined conditions.

Of the 24 whose gas ratio falls between 1.5 and 2, 15 form from 10 to 13.9 c.c. of gas and nine from 14 to 15.9 c.c. of gas. Thirty-five cultures have a gas ratio above 2 and of these 23, or 65 per cent, form between 14 and 15.9 c.c. of gas, while of the remaining 12 cultures, 6 are slightly above this amount and 6 slightly below. This relation of the gas ratio to the amount of gas formed has already been pointed out and is repeated here merely to show the distinct correlation between these characters.

We have then as the beginning of one subgroup 65 cultures forming less than 10 c.c., almost always 7–8 c.c., of gas with a CO_2 to H_2 ratio of approximately 1.1. If these two characters mark a

natural group we should find that these cultures have in common a number of other characters, either positive or negative, not possessed in common with the other cultures of our collection.

For this purpose we may use to advantage the fermentation of various test substances. Winslow in his work on the coccaceae has used the amount of acid formed to good advantage in separating strains, but with gas formers this is of less value on account of the frequent partial or complete neutralization of the acid in the later part of the incubation. However, we have tabulated the frequency of occurrence for certain arbitrary amounts of acid formed.

Practically all of the cultures ferment dextrose, levulose, galactose, salicin, and lactose. These fermentations are therefore characteristic of the entire group and are of no value in forming subdivisions. Inulin is fermented by so very few that, so far as our cultures are concerned at least, it is of no value. The curve for nearly all of these substances, excepting those in which the mode is obscured by the secondary alkali formation, indicates that the demarkation between fermentation and no fermentation falls at about 0.1 per cent lactic acid. This point has been used in constructing the subsequent tables for all the test substances with the exception of glycerin in which the low point falls at 0.2 per cent. The correctness of this deduction is supported by the fact that no correlation could be found between the fermentation of glycerin and other reactions when the separation was made at 0.1 per cent, while when the division was made at 0.2 per cent certain correlations, which will be pointed out later, became quite evident. If the group of cultures giving a gas ratio of approximately 1.1 is a natural group it should be distinguished from the remaining gas-forming cultures by the possession of several characters not common to the entire collection.

An examination of the tables giving the fermentation of the various test substances shows that the group of cultures with the gas ratio below 1.4 has a distinctly lower fermentative ability than the remaining cultures. This is illustrated by Fig. 10, which shows the relative number of test substances fermented by each group. The average number of the low ratio group, represented by the solid line, ferment 8 or at the most 10 of the 13 substances,

while those with a higher ratio ferment from 9 to 12 of the test substances.

If we consider each of the significant characters, a comparison of the two groups shows that the distinction between them is well defined and specific. This is shown in Table 14, p. 460.

FIG. 10.—Relative number of test substances fermented by different groups.

The difference in the action on nitrates and in the production of indol is not great but shows a slightly higher activity for the low ratio group. With adonite the difference is much in favor of the high ratio group. The high ratio group is more active in the

fermentation of saccharose and raffinose and with starch the difference is very marked. Dulcite and glycerin are fermented with difficulty by most bacteria and we should expect that only a very few cultures of the low ratio group would be able to ferment these two substances. On the contrary, 50 per cent of the low ratio

TABLE 14.

CORRELATION OF CO_2 : H_2 RATIO WITH PHYSIOLOGICAL REACTIONS.

Ratio	Total Cultures	Indol		Nitrates		Adonite		Saccharose		Raffinose		Starch		Dulcite		Glycerin		Gelatin	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Below 1.4....	58	51	7	56	2	9	48	23	35	25	33	3	55	29	29	33	25	2	55
Percentage...	.....	87.9	12.1	96.5	3.5	15.8	84.2	39.7	60.3	43.1	56.9	5.2	94.8	50.0	50.0	56.9	43.1	3.5	96.5
Above 1.4....	56	34	22	51	5	31	25	54	2	51	5	34	22	15	41	10	44	10	42
Percentage...	.....	60.7	39.3	91.0	9.0	55.3	44.7	96.4	3.6	91.0	9.0	60.7	39.3	26.8	73.2	18.5	81.5	19.2	80.8

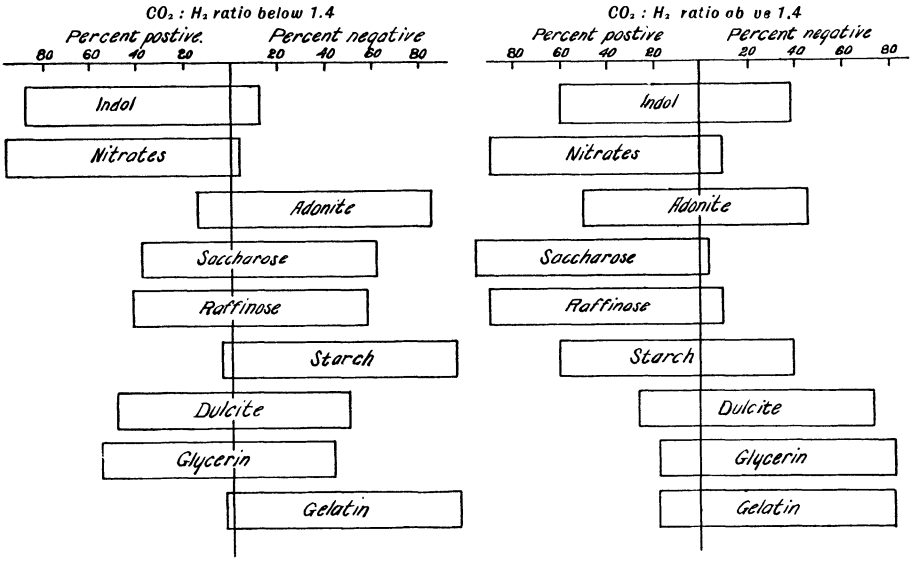


FIG. 11.—Correlation of CO_2 : H_2 ratio with physiological reactions.

cultures ferment dulcite and 57 per cent ferment glycerin, while of the high ratio group 27 per cent ferment dulcite and 18 per cent ferment glycerin. Only 12 cultures liquefy gelatin and of these 10 belong to the high ratio group.

The differences between these groups may be made more clear by reference to Fig. 11 in which the frequency of occurrence of the

significant characters is represented graphically. The number of cultures in each group giving positive reactions with the different tests is plotted to the left of the line, while those with a negative reaction are arranged on the right of the line. This, we believe, shows very clearly that the gas ratio of approximately 1.1 with its correlated characters marks a well defined natural subgroup of the so-called colon-aerogenes group.

It is not improbable that the application of similar methods would result in a more minute division of the group already established. A possible basis for further differentiation may be found in the dulcite fermentation which is positive in one-half of the cultures of the low ratio group. Comparing the fermentative ability of those cultures which ferment dulcite with those that do not, we obtain Table 15.

TABLE 15.

CORRELATION BETWEEN DULCITE FERMENTATION AND OTHER CHARACTERS.

Dulcite	Number of Cultures	Indol		Nitrates		Adonite		Saccharose		Raffinose		Starch		Gylcerin	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
+	29	26	3	28	1	0	28	13	16	14	15	0	29	14	15
Percentage of total		89.66	10.34	96.55	3.45	0	100	44.83	55.17	48.28	51.72	0	100	48.28	51.72
−	29	25	4	28	1	9	20	11	18	11	18	3	26	17	12
Percentage of total		86.20	13.80	96.55	3.45	31.03	68.97	37.93	62.07	37.93	62.07	10.34	89.66	58.62	41.37

In indol production and the reduction of nitrates the two groups do not differ. None of the dulcite fermenters have any action on adonite but they show a somewhat higher activity in fermenting saccharose and raffinose than the cultures giving a negative reaction with dulcite.

Only three cultures of the entire group act on starch and these belong with those failing to ferment dulcite. The dulcite negative group is somewhat more active in fermenting glycerin than the dulcite positive group. While the relation between dulcite and adonite fermentation is striking, the correlation between dulcite fermentation and other characters is hardly strong enough to warrant the assumption of a natural division on results obtained from a comparatively small number of cultures.

TABLE 16.

CORRELATION OF GAS RATIOS ABOVE 1.4 WITH PHYSIOLOGICAL REACTIONS.

Ratio	Number of Cultures	C.C. Gas from 5 c.c. Broth			Indol		Nitrates		Adonite		Saccharose		Raffinose		Starch		Glycerin		Dulcite		Gelatin	
		10–13.9	14–15.9	16 Up	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–
1.5–2	23	14	9	0	17	6	21	2	13	10	21	2	22	1	14	9	5	18	5	18	4	17
Percentage of total		60.87	39.13	0	73.91	26.09	91.30	8.70	56.52	43.48	91.30	8.70	95.65	4.35	60.87	39.13	21.74	78.26	21.74	78.26	19.05	80.95
Above 2	35	6	24	5	21	14	32	3	20	15	32	3	31	4	19	16	11	24	11	24	3	32
Percentage of total		17.14	68.57	14.29	60.00	40.00	91.43	8.57	57.14	42.86	91.43	8.57	88.57	11.43	54.28	45.71	31.43	68.57	31.43	68.57	8.57	91.43

The striking correlations found in the *B. communis* group, as shown in Table 10, are readily understood when one compares them with the distribution of reactions exhibited in Table 14.

Practically all cultures coming under the arbitrary dulcite positive and saccharose negative combination belong in the low ratio group but this method of classification splits up the group based on the gas ratio, which we have every reason to believe a natural one. Any correlations which may occur are accidental and are offset by the lack of correlation in species produced by other combinations of characters.

THE HIGH RATIO GROUP.

The subdivision of the high ratio group presents a much more difficult problem. Natural bacterial groups, like the natural groups of higher organisms, are produced by long existence under uniform conditions; in other words, are closely associated with a definite habitat. Milk is not the habitat of the gas-forming bacteria. On the contrary, the bacteria in milk represent the sum of the contamination of the milk, and consequently include, under the ordinary conditions, a large number of varieties. Our collection of cultures giving ratios above 1.4 doubtless includes representatives of many groups whose limits so overlap that they could not be separated with any certainty without additional study on a larger collection. We have seen also that the gas

ratio, which in the group just considered indicated the principal line of demarkation, becomes more variable as the ratio becomes higher and the reaction more complicated.

Fig. 8 shows some evidences of a separation on the basis of the gas ratio at about 2.0–2.1. The correlation of the two groups made by this somewhat arbitrary division is given in Table 16.

The relation between the ratio and the amount of gas which has already been pointed out holds to some extent with these two groups but beyond this there are no differences which could be considered as distinctive. On the contrary, the percentage of positive reactions for each of the various tests show a surprising similarity especially when they are contrasted with some of the preceding tables in which a distinction between two groups can be made.

Included in the high ratio group are 10 cultures which liquefy gelatin. When these cultures are arranged as in Table 17 so that

TABLE 17.

CORRELATION OF GELATIN LIQUEFACTION WITH FERMENTATIONS.

Cultures with CO_2:H_2 Ratio above 1.4.

Gelatin Liquefied	Number of Cultures	Ratio 1.7–2.2		Adonite		Saccharose		Raffinose		Starch		Glycerin		Dulcite	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−
+	10	9	1	2	8	10	0	7	3	6	4	7	3	1	9
Percentage	.....	90	10	20	80	100	0	70	30	60	40	70	30	10	90
−	42	15	27	28	14	39	3	41	1	26	16	3	39	12	30
Percenatge	.....	35.71	64.29	66.66	33.33	92.86	7.14	97.62	2.38	38.09	61.91	7.14	92.86	28.58	71.42

they may be compared with other high ratio cultures which do not liquefy gelatin, we find that they have in common certain characters which tend to separate them from the non-liquefiers. The ratio of 9 of the 10 cultures is identical within narrow limits; nearly all of them fail to ferment adonite and dulcite; they all ferment saccharose and over 60 per cent of them ferment raffinose, starch, and glycerin. The percentage of raffinose fermenters is much higher in the non-liquefiers but the reverse is true in the case of starch and glycerin. A similar relation between the liquefaction of gelatin and the fermentation of glycerin was observed in the streptococci.

CULTURES WITH UNUSUAL GAS PRODUCTION.

In addition to the organism whose gas production has been discussed we have still to describe the gas production of several bacteria which could not be included in Table 9 because they produce no gas in broth containing dextrose. These cultures were isolated from pasteurized milk and are described in a paper by Ayers and Johnson of this laboratory. The conduct of these bacteria when grown in vacuum bulbs shows them to be distinct in many ways from those whose gas production has been described in the preceding pages, and to constitute a very distinct group. The peculiarities which they display cannot be intelligently discussed until further data are obtained. The analyses made will simply be presented for whatever use they may be found to have for purposes of classification and without further comment.

TABLE 18.

THE GAS PRODUCTION OF *fa* IN BROTH.

5 c.c. Standard Broth, 1 Per Cent Dextrose Incubated 7 Days at 30° C. Abundant Growth.

Total Gas	CO_2
c.c.	%
0.32	85.0
0.68	80.0

5 c.c. Standard Broth, 1 Per Cent Lactose Incubated 7 Days at 30° C.

Total Gas	CO_2	H_2
c.c.	%	
1.06	56	Present
1.05	59	"

TABLE 19.

THE GAS PRODUCTION OF *fa* IN 5 C.C. MILK AT 30° C. VACUUM BULB.

Period of Incubation	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
Days	c.c.	c.c.	c.c.	%	%	
7	24.5	14.50	6.66	59.2	27.2	2.18
14	44.07	27.43	16.45	62.2	37.3	1.67
21	28.74	17.18	11.48	59.8	39.9	1.50
31	23.37	13.80	9.56	59.0	40.9	1.44
43	48.36	30.58	17.00	62.9	34.9	1.80
47	45.29	28.25	16.83	62.4	37.2	1.68
47	43.31	27.24	15.93	62.9	36.8	1.71
80	49.28	31.04	18.18	63.0	36.9	1.71

TABLE 20.

GAS PRODUCTION OF *fm*.

5 c.c. Standard Broth, 1 Per Cent Dextrose. No Growth. Duplicates.
5 c.c. Standard Broth, 1 Per Cent Mannite, Incubated 15 Days at 30° C. Vacuum Bulb.

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
21.60.....	10.85	10.61	50.2	49.1	1.02

5 c.c. Milk Incubated 17 Days at 30° C. Vacuum Bulb.

Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
c.c.	c.c.	c.c.	%	%	
42.36.....	25.93	16.43	61.2	38.8	1.58

The slowness with which the gas was produced by this organism when grown in Smith tubes led us to try experiments with large quantities of milk in vacuum with the view of following the course of the reaction from day to day. Accordingly a bulb was prepared holding 200 c.c. of skim milk. After sterilization and inoculation it was evacuated, and then the gas evolved each day was pumped out as completely as possible and analyzed. The enormous amount of gas which was produced each day was more than the pump could manage successfully. Consequently we will omit the analyses, and simply state that at the end of 500 hrs., when the experiment was discontinued, 3,069.9 c.c. of gas had been collected.

A second experiment conducted with 50 c.c. of milk was followed more accurately and this we report below:

TABLE 21.

GAS PRODUCTION BY *fm* FROM 50 C.C. MILK AT 30° C.

Hours	Total Gas to Date	Total Gas	CO_2	H_2	CO_2	H_2	Ratio CO_2/H_2
	c.c.	c.c.	c.c.	c.c.	%	%	
6.....	0.14	0.14	.....	.....	.....	.....	.....
30.....	0.25	0.11	.....	.....	.....	.....	.....
48.....	17.80	17.55	10.66	6.87	60.8	39.1	1.55
54.....	35.76	17.96	10.18	7.76	56.7	43.2	1.31
99.....	190.26	154.50	91.16	70.35	59.0	41.0	1.44
121.....	289.57	99.31	59.74	39.57	60.2	39.8	1.51
170.....	484.97	195.40	114.70	80.70	58.7	41.3	1.42
193.....	591.77	106.80	62.82	43.98	58.8	41.2	1.43
217.....	685.87	94.10	53.80	40.30	57.2	42.8	1.33
242.....	772.67	86.80	50.00	36.80	57.6	42.4	1.36
266.....	852.51	79.84	47.90	31.90	59.9	40.1	1.50
289.....	926.62	75.11	44.00	31.00	58.6	41.4	1.42
337.....	1,053.62	127.00	73.40	53.60	57.8	42.2	1.37
362.....	1,141.18	87.56	50.63	36.90	57.8	42.2	1.37
385.....	1,214.80	73.62	42.30	31.30	57.4	42.6	1.35
409.....	1,256.64	41.84	22.70	19.10	54.3	45.7	1.19
433.....	1,266.11	9.47	5.50	3.90	58.9	40.2	1.47
457.....	1,268.60	2.45	1.89	0.60	76.0	24.0	3.16
505.....	1,270.10	1.30	0.95	0.35	72.8	26.7	2.73
553.....	1,271.20	1.30	0.68	0.42	61.8	38.2	1.62
601.....	1,272.45	1.25	0.70	0.55	56.0	44.0	1.27

In Fig. 12, are plotted the total volumes of gas up to the end of each period as ordinates against hours as abscissae.

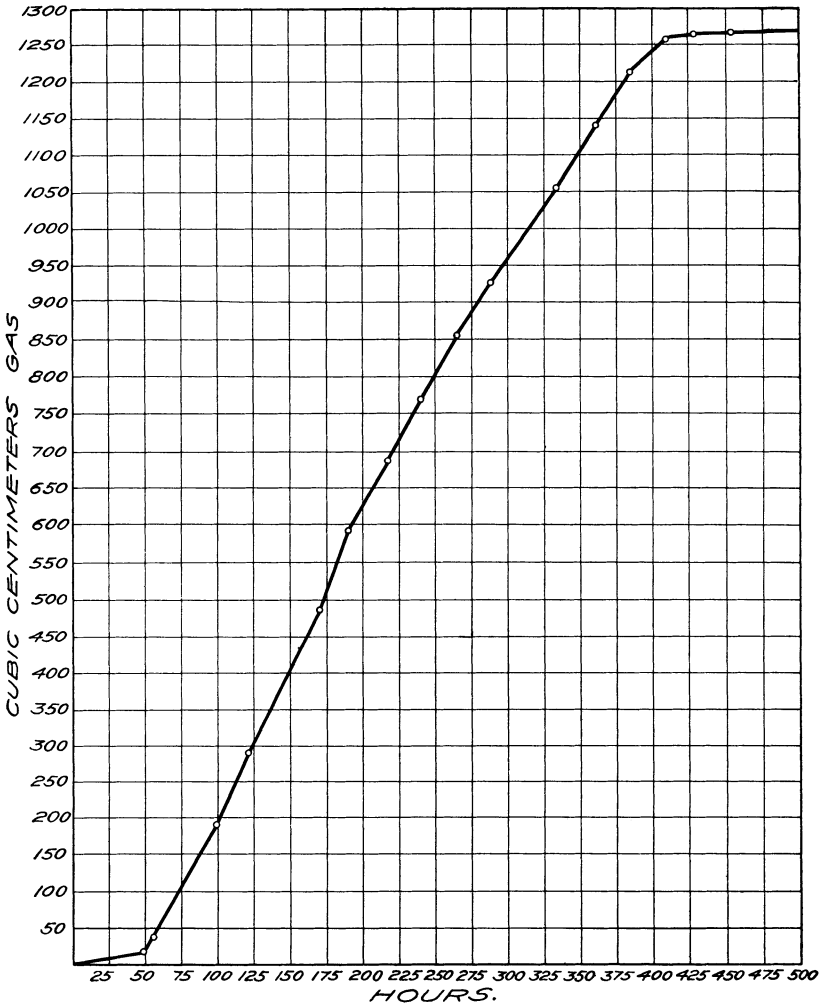


FIG. 12.—Gas production by *fm* from 50 c.c. milk.

CONCLUSIONS.

In drawing conclusions from the data presented, one fact stands out above all others, that certain bacteria when cultivated in dextrose broth at 30° C. furnish a total quantity of gas and a ratio

of the constituent gases which when accurately determined are not found to be remarkably constant. These bacteria find themselves in our Group I with a ratio $\frac{CO_2}{H_2}$ of approximately 1:1.

Other cultures of gas-producing bacteria furnish quantities of gas and ratios of carbon dioxid to hydrogen which, while they are not so constant as those of the first group, are yet clearly distinctive.

Among the cultures furnishing high ratios of $\frac{CO_2}{H_2}$ a few appear distinct because of exceptionally high ratios. Whether these may safely be made a group by themselves on the basis of their gas production alone cannot be said until further data are obtained.

One of the organisms is distinguished by forming carbon dioxid alone and others are distinguished both by qualitative differences in their ability to form gas by fermentation of sugars and by the slow rate of gas evolution.

We have found in the increased constancy and reproducibility of both volumes of gas and ratios of constituent gases, discovered by the substitution of accurate for inaccurate methods of isolation and analysis, a strong suggestion that, just as the older methods furnished discrepant data through their inaccuracies, so the discrepancies of the present data may be traced to the inability of the method to furnish information upon anything but the end products. We have presented a few reasons to support the hypothesis that in those cases where we obtained closely agreeing data we were indeed analyzing the end products of a single reaction while in the more discordant cases we caught only the end products of two or more reactions progressing at different rates. The justification of this assumption is the hope that by further penetration we may be able to discover means of confining the action of the bacteria and obtain but one gas-producing reaction whose end products will appear in the analytical data with the constancy and consequent diagnostic value found in the data for our Group I.

Aside from this the data of the gas determination when given face value appear only to separate our cultures into distinct groups and we have used these as the basis of correlation.

To what extent are we justified in rearranging the group or

establishing new descriptions for species already described? The rule of the botanists and zoölogists of holding to the original description cannot be followed strictly in bacteriology on account of the frequent inaccuracy and insufficiency of the earlier descriptions and even of many of those of later date. Escherich's description of *B. coli communis* was based on culture characters that have been gradually discarded until they are no longer found in the most recent descriptions. So many writers and committees have described this organism, differing one from another, in the characters which are to be considered as significant or as unimportant that the limits of the species, if we may be permitted to call it a species, may be looked upon as a movable function. We find that the ratio of hydrogen to carbon dioxid may vary within wide limits; that *B. coli* always forms indol or that indol formation is unimportant; that it never liquefies gelatin or that it may liquefy gelatin; that it always ferments dulcite or that it occasionally ferments dulcite, and so on. We think, therefore, that we may be permitted to add our version of the salient characters of *B. coli* to the many already published. It should be understood, however, that we make this merely as a suggestion to be used as the basis of further work and not as a final statement of the characters to which *B. coli* must conform. In our opinion the sharply defined group characterized particularly by a gas ratio of approximately 1:1 should be considered, on account of its frequent occurrence and its agreement with the more authentic descriptions, to be *B. coli* in its narrower sense. We may, then, describe *B. coli* as a short, thick rod, gram negative, and usually but not uniformly motile. Indol is produced by nearly all cultures and nitrates are usually reduced to nitrites. Under anaerobic conditions reduction of neutral red almost always takes place. Dextrose is always fermented with the formation of carbon dioxid and hydrogen in nearly equal parts. From 5 c.c. of 1 per cent dextrose broth 6–8 c.c. of gas are produced. Adonite is seldom fermented; lactose, galactose, and levulose are always fermented but many cultures fail to ferment saccharose and raffinose. Starch is rarely fermented but about one-half of the cultures ferment dulcite and glycerin. Gelatin is very rarely liquefied. It grows well on agar and other artificial media. It

is not improbable that two quite distinct varieties exist, one fermenting dulcite and usually failing to ferment adonite and the other failing to ferment dulcite but frequently fermenting adonite. These may be taken to correspond to the old varieties, *communior* and *communis*, but the evidence is not yet sufficient to warrant definite statements.

Closely related to *B. coli* is a group distinguished from it by a marked difference in the gas ratio, which indicates a more complicated reaction, and by a greater fermentative ability in general. This is shown not only in the number of substances fermented and the volume of gas formed but also in the percentage of cultures fermenting the more complex carbohydrates. This difference is especially noticeable in saccharose and raffinose, which are fermented by nearly all cultures, and in adonite and starch, which are fermented by over 50 per cent of the cultures. On the other hand, only a very few ferment dulcite and glycerin. While it is evident that this is a heterogeneous group, we have not yet collected sufficient information to warrant us in making subdivisions. There is some indication of a group formed about the liquefaction of gelatin, but as there were only 10 liquefying high ratio cultures generalizations are unsafe.

It should be noted, however, that these cultures have many characters in common which differentiate them from the remaining high ratio cultures.

While this work has not been sufficient to warrant a complete revision of the colon-aerogenes group, we believe that it marks out the lines along which investigations must proceed if a revision is to be made that will stand the test of time. We need to know, most of all, the mechanism of the reaction which results in the evolution of hydrogen and carbon dioxid, the steps by which the fermentation proceeds, and the products formed in the process. We should know definitely the nature and the source of the by-products causing the alkalin reaction which frequently follows the gaseous fermentation.

The chemistry of the Vogues and Proskauer reaction should be studied until the test can be made accurately and under exact conditions.

When acquired, this information must be so applied that the significant characters can be determined and used to establish a classification that will be lasting because it will separate the group into species made by nature and not by a committee of bacteriologists.

SUMMARY.

A collection of typical gas-forming bacteria occurring in milk was obtained from widely separated and representative sources.

These cultures were examined for morphology, spore formation, Gram-stain, amount of gelatin liquefaction, production of indol, reduction of nitrates and neutral red, and amount of acid produced from dextrose, levulose, galactose, adonite, saccharose, lactose, raffinose, starch, inulin, mannite, glycerin, salicin, and dulcite. Particular attention was given to the production of gas in media containing dextrose, and for the isolation of this gas the exact method of Keyes with some modifications was used.

The carbon dioxid to hydrogen ratio which occurred with the greatest frequency was approximately 1:1. Plotted on the frequency basis this ratio stands apart from all higher ratios.

All cultures giving the 1:1 ratio are distinguished from high ratio cultures by the amount of gas formed under exact conditions. This was uniformly less than the amount produced under identical conditions by the high ratio cultures.

The amount of acid produced from the individual test substances could not be used to advantage because this was frequently obscured by a secondary alkalin fermentation in which the acid was partially or entirely neutralized.

The low ratio cultures fermented a smaller number of test substances than those giving a high ratio, but this difference was not always in those substances usually considered to be fermented with the greater difficulty.

The low ratio cultures are distinguished by a high percentage of cultures giving a positive indol test, reduction of nitrates, and fermentation of dulcite and glycerin. Very few cultures ferment adonite and starch, and only about 40 per cent ferment saccharose and raffinose. The high ratio cultures, on the other hand, give a lower percentage of positive tests with indol, a much higher number

of positive tests with adonite and starch, nearly all ferment saccharose and raffinose but only a very few ferment dulcite and glycerin.

The low ratio group may possibly be divided into those which ferment dulcite but fail to ferment adonite and starch, and those which do not ferment dulcite but occasionally ferment adonite and starch.

No data are available for a logical subdivision of the high ratio group with the possible exception of the small number liquefying gelatin.

The 10 gelatin liquefying cultures agreed very closely in the gas ratio, the fermentation of saccharose and glycerin, and the failure to ferment adonite and dulcite. They differed from other high ratio cultures especially in the gas ratio and the fermentation of glycerin.

The collection contained a few cultures which differed radically from the others in giving a prolonged fermentation in milk with the production of enormous quantities of gas, and one type which was distinguished by the fermentation of dextrose with the production of carbon dioxid only.

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